DISSERTATION

POTATO AND GRAPE POLYPHENOLS, RESPECTIVELY, SUPPRESS HIGH-FAT DIET-ELEVATED OXIDATIVE STRESS/INNATE INFLAMMATION MARKERS IN PORCINE MODEL AND INDUCE APOPTOSIS IN HCT-116 P53 +/+ AND P53 -/- HUMAN COLON CANCER CELL LINES IN VITRO

Submitted by

Sridhar Radhakrishnan

Department of Food Science and Human Nutrition

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2014

Doctoral Committee:

Advisor: Jairam Vanamala

Sung Woo Kim
Christopher Melby
Michael Pagliassotti
DN Rao Veeramachaneni

Copyright by Sridhar Radhakrishnan 2014

All Rights Reserved

ABSTRACT

POTATO AND GRAPE POLYPHENOLS, RESPECTIVELY, SUPPRESS HIGH-FAT DIET-ELEVATED OXIDATIVE STRESS/INNATE INFLAMMATION MARKERS IN PORCINE MODEL AND INDUCE APOPTOSIS IN HCT-116 P53 +/+ AND P53 -/- HUMAN COLON CANCER CELL LINES IN VITRO

Rationale

***In Vitro* Study**

Bioactive compounds from fruits and vegetables have demonstrated anti-inflammatory, anti-oxidant and anti-cancer properties. Grapes and purple-fleshed potatoes are rich sources of polyphenols. Grape seed extract (GSE) a popular dietary supplement rich in proanthocyanidins, demonstrated anti-cancer properties in a variety of *in vitro* and *in vivo* models. Red grapes are rich in resveratrol (RSV), a compound that has shown anti-oxidant, anti-inflammatory and anti-cancer properties in a variety of models including human studies. We previously reported that RSV suppressed proliferation, and induced apoptosis via p53 activation in human colon cancer cell lines HT-29 and SW-480, however, only at high concentration > 75-100 µM. Since bioactive compounds exist as a complex mixture in fruits and vegetables with synergistic or additive chemopreventive/protective actions, we hypothesized that the combination of RSV and GSE would be a more potent mixture with efficacy at lower concentrations. We tested anti-cancer efficacy of RSV and GSE, alone and in combination, in HCT-116 p53 +/+ and -/- human colon cancer cells. We tested anti-cancer efficacy of RSV and GSE in the presence of IGF-1, a mitogen elevated during obesity.

Animal Study

High-fat diet (HFD) consumption is linked to elevated risk for a variety of disorders. HFD elevated oxidative stress and inflammation and provides conducive environment for chronic disorders such as colon cancer. Dietary modification can help reduce risk for such conditions. Indeed, higher consumption of bioactive compounds present in fruits and vegetables are inversely associated with risk for such inflammatory disorders. The potato is the third largest source of phenolic compounds in the human diet after oranges and apples. Purple-fleshed potatoes are rich in anthocyanins and phenolic acids with anti-oxidant and anti-inflammatory properties. Unlike other anthocyanin-rich sources such as blueberries, purple-fleshed potatoes are relatively inexpensive and widely available, but they are almost always consumed in processed form. Thus, it is important to understand the effect of processing on purple-fleshed potato bioactive compounds before we present it as a functional food – a delivery vehicle for health promoting bioactive constituents. The overall goal of these studies was to determine the extent to which purple-fleshed potato consumption, even after processing, prevent or reverse HFD elevated oxidative stress and innate inflammatory markers (colon, mesenteric fat and systemic circulation) in a pig model.

Animal Study Experiment Design

We hypothesized that consumption of purple-fleshed potatoes, even after processing, will suppress HFD elevated colonic, mesenteric fat and systemic oxidative stress/inflammation markers in the pig model compared to white-fleshed potatoes and HFD control. To test this hypothesis, we performed two studies – a prevention study where 64 pigs, 3 weeks post-weaning, consumed one of the eight diets: standard diet (SD), HFD and HFD supplemented with

raw, baked or chipped purple or white-fleshed potatoes (10 % w/w) for 13 weeks; and a reversal study where pigs (12 weeks on HFD) consumed HFD containing 10/20 % purple or white-fleshed potato chips for additional 5 weeks (n = 8).

Results and Conclusions

In Vitro Study

Our results demonstrated that RSV (~ 25 µM) and GSE (35-50 µg/ml) mixture is potent in suppressing proliferation and elevating apoptosis in HCT-116 p53 +/+ human colon cancer cell lines at lower concentrations compared to RSV or GSE alone. RSV potentiated GSE induced p53 dependent apoptosis via mitochondrial apoptotic signaling as evidenced by elevated Bax:Bcl-2 ratio, reactive oxygen species and activated caspase-3. The RSV-GSE combination suppressed proliferation and induced apoptosis even in presence of IGF-1 (elevated during obesity), suggesting its potential role as a chemopreventive agent against IGF-1 promoted colon cancer. Moreover, RSV-GSE did not suppress proliferation or induce apoptosis in normal colonic epithelial cell line CRL-1831, demonstrating its specificity to cancer cells. These results strongly support our hypothesis that combining bioactive compounds like RSV and GSE could provide similar or better anti-colon cancer properties compared to individual compounds at lower concentrations. This lends support to the proposal that combinatorial approach towards colon cancer chemoprevention using bioactive compounds is a feasible approach. However, animal studies using such a RSV-GSE combination is warranted.

Animal Study

In the prevention study, only the purple-fleshed potato raw group had significantly lower distal colonic and mesenteric fat oxidative stress (measured using GSH:GSSG ratio, a sensitive marker for oxidative stress) compared to the HFD control. However, all the potato diets consuming animals had suppressed ($P \leq 0.05$) distal colonic and mesenteric fat expression of innate inflammatory markers TLR-4, NF-κB, and TNF-α compared to the HFD control and similar to that of SD control animals. Animals consuming potato diets had significantly suppressed urinary 8-isoprostane (8IP) and DNA adduct 8-OHDG (Enzyme immunoassay), and serum TNF-α (ELISA) and IL-1β (Milliplex immunoassay) compared to the HFD control animals. In the reversal study, the markers of distal colonic and systemic oxidative stress and inflammation were consistently suppressed only in the purple-fleshed potato consuming groups compared to HFD controls ($P \leq 0.05$), suggesting that time of intervention is very important. Results from both studies show that only the purple-fleshed potato, even after processing, prevented and reversed HFD elevated oxidative stress/inflammation markers in the distal colon, mesenteric fat and systemic circulation, in the pig – a human relevant animal model. Thus, purple-fleshed potatoes can serve as a great delivery vehicle for anti-inflammatory bioactive compounds.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Jairam Vanamala for seeing potential in me and giving me the chance to work in his laboratory to pursue my graduate studies. His encouragement, guidance and timely advice helped me accomplish this great task. His passion for science made me push harder each day and complete my work with dedication. I am thankful for his help in becoming a matured individual along with being a better scientist.

I cannot really express how grateful I am to Dr. Lavanya Reddivari. She has been always ready to help in spite of her busy schedule. She taught me most of the technical skills in the laboratory for me to be successful. She is a crucial element in this thesis and was actively involved right from experimental designing till writing up the results. Although, she is not my committee member, she has helped me like one, providing me feedback all through my doctoral program.

I thank all my lab members over the years, Gaurav Madiwale, Venkata Charepalli, Fauzi Amer, Aaron Massey, Emily Poole, Andy Kester, Orlando Perez, Birunda Charles, Xia Xiao, Amulya Balumuri, Amber Kahl and Laura Markham for their help with the study and their awesome company in the lab.

I thank Dr. Sung Woo Kim for his help and support through the animal studies performed, I am sure it would have been impossible to conduct my studies without his supervision and impeccable organization skills. I thank members of Dr. Kim lab, Jiyao Guo, Alexandra Chaytor, Leah Jones, Justin Parker, Yan Bin Shen, Gwendoline Voilque, Adsos Passos and Yan Zhao for the help during the animal study.

I thank my other committee members, Dr. Michael Pagliassotti, Dr. Christopher Melby and Dr. DN Rao Veeramachaneni for their intellectual input, feedback on my dissertation and support for this work.

I thank Dr. David Holm, Black Gold Farms, TX, H.E.B., TX, Kettle Chips, OR, and Vandrunen Farms, IL, for their help to obtain potatoes and process them for the animal studies. Special thanks for all people at the potato processing plant who were involved in processing and packing potato material for the study. Thanks to staff of the Swine Education Unit and Swine Evaluation Station, North Carolina State University for their resources and provide me training to work with the pig model. Special thanks to Carl Whisenant, North Carolina State University Feed Mill, who helped me make diets for the study. I thank Chris Mulligan, Department of Food Science and Human Nutrition, and Corey Broeckling, Department of Microbiology at Colorado State University, for their help with my experiments. I wish to thank every member of the Department of Food Science and Human Nutrition, CSU for their continuous help and support at different periods of my work. A special thanks to the staff of the Department.

I thank the USDA-NIFA, as my PhD was supported by National Research Initiative Grant 2009-55200-05197 funded to my advisor, Dr. Jairam Vanamala.

I would like to thank all my friends from India, Colorado State University, Texas A&M University and from North Carolina State University for making my time during PhD a memorable one. Heartfelt thanks for those whom I have missed to acknowledge.

DEDICATION

This dissertation is dedicated to my parents Mr. G. Radhakrishnan and Mrs. T. A. Annapurni whose unconditional love and support made this possible. They have instilled in me the importance of hard work and higher education right from childhood and I dedicate this work to their unwavering support, their patience and understanding.

I also dedicate this work to my family members and friends in India and the USA for their constant words of encouragement.

I dedicate this dissertation to almighty god for helping me through difficult times.

Finally, this dissertation is dedicated to all those who believe in the richness of learning.

SRIDHAR RADHAKRISHNAN

TABLE OF CONTENTS

ABSTRACT…………………………………………………………………………... ii

Rationale…... ii

In *Vitro* Study………………………………………………………………… ii
Animal Study…………………………………………………………………. iii

Results and Conclusions……………………………………………………… iv

In *Vitro* Study………………………………………………………………… iv
Animal Study…………………………………………………………………. v

ACKNOWLEDGEMENTS…………………………………………………... vi

DEDICATION………………………………………………………………... viii

LIST OF TABLES……………………………………………………………. xiv

LIST OF FIGURES……………………………………………………………... xv

CHAPTER I: INTRODUCTION……………………………………………... 1

1.1 Aims………………………………………………………………………. 3
1.2 References……………………………………………………………….... 5

CHAPTER II: REVIEW OF LITERATURE………………………………… 8

2.1 General Overview of Bioactive Compounds……………………………... 8
2.2 Colon Cancer……………………………………………………………... 9
2.3 Resveratrol………………………………………………………………... 10
2.4 Grape Seed Extract……………………………………………………….. 11
2.5 Synergy of Bioactive Compounds………………………………………... 12
2.6 Aim 1……………………………………………………………………... 14
2.7 Diet and Oxidative Stress/Inflammation…………………………………. 15
2.8 The Potato………………………………………………………………… 18
2.9 Potato Processing…………………………………………………………. 22
2.10 Pig as a Model…………………………………………………………… 23
2.11 Aim 2.…………………………………………………………………… 24
2.12 References………………...…………………………………………….. 26

CHAPTER III: RESVERATROL POTENTIATES GRAPE SEED EXTRACT INDUCED HUMAN COLON CANCER CELL APOPTOSIS VIA ACTIVATION OF P53-DEPENDENT SIGNALING PATHWAYS………………………………………….. 41

3.1 Summary………………………………………………………………………….. 41

3.2 Introduction……………………………………………………………………….. 42

3.3 Materials and Methods…………………………………………………………… 45
3.3.1 Chemicals………………………………………………………………………….. 45
3.3.2 LC-MS Analysis of Grape Seed Extract……………………………………….... 47
3.3.3 Cell Lines………………………………………………………………………….. 48
3.3.4 Cell Proliferation Assay…………………………………………………………… 49
3.3.5 Cytotoxicity Detection…………………………………………………………….. 49
3.3.6 Apoptosis Assay…………………………………………………………………... 50
3.3.7 Experimental Design of Combination Study……………………………………… 50
3.3.8 Fluorescence Activated Cell Sorting Analysis…………………………………… 56
3.3.9 Western Blot Analysis…………………………………………………………….. 56
3.3.10 IGF-1 Treatment………………………………………………………………… 57
3.3.11 Statistical Analysis……………………………………………………………….. 58

3.4 Results…………………………………………………………………………….. 58
3.4.1 Resveratrol (RSV) or Grape Seed Extract (GSE) Suppressed Cell Proliferation of HCT-116 Cells…………………………………………………………………………... 58
3.4.2 RSV or GSE Induced Apoptosis…………………………………………………... 59
3.4.3 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis….. 59
3.4.4 RSV-GSE Combination Induced Cell Cycle Arrest in Go/G1 Phase in Both HCT 116 P53 +/+ and P53 -/- Cells……………………………………………………………… 60
3.4.5 RSV-GSE Combination Induced Apoptosis is P53-Dependent…………………... 61
3.4.6 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis via Caspase-3 Dependent and Bax/Bcl-2 Pathways…………………………………………… 64
3.4.7 N-Acetyl Cysteine (NAC) Inhibited RSV-GSE Combination Induced Apoptosis in HCT-116 P53 +/+ Cells………………………………………………………………… 67
3.4.8 RSV-GSE Combination is Non-Toxic to Normal Cells…………………………... 68
3.4.9 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis Even in the Presence Of IGF-1……………………………………………………………….. 70

3.5 Discussion………………………………………………………………………… 71

3.6 References………………………………………………………………………… 78

CHAPTER IV: PURPLE-FLESHED POTATOES REVERSED HIGH-FAT DIET ELEVATED OXIDATIVE STRESS AND INNATE INFLAMMATION MARKERS IN A PIG MODEL………………………………………………………………… 85

4.1 Summary……………………………………………………………………….. 85

4.2 Introduction…………………………………………………………………….. 86

4.3 Materials and Methods…………………………………………………………. 90
4.3.1 Animals………………………………………………………………………. 90
4.3.2 Experimental Diets………………………………………………………….... 90
4.3.3 Blood, Urine and Tissue Collection………………………………………….. 94
4.3.4 Tissue Oxidative Stress………………………………………………………. 94
4.3.5 Real Time Polymerase Chain Reaction…………………………………… 94
4.3.6 Systemic Oxidative Stress Markers………………………………………….. 97
4.3.7 Serum Inflammatory Markers……………………………………………….... 97
4.3.8 Statistical Analysis……………………………………………………………. 97

4.4 Results………………………………………………………………………….. 98
4.4.1 UPLC-MS Profile of Phenolic Compounds in Potato Chips……………………… 98
4.4.2 Physical Parameters………………………………………………………….. 102
4.4.3 Colonic GSH:GSSG Ratio……………………………………………………. 105
4.4.4 Colonic Inflammatory Markers………………………………………………… 106
4.4.5 Mesenteric Fat GSH:GSSG Ratio…………………………………………….. 108
4.4.6 Mesenteric Fat Inflammatory Markers……………………………………… 109
4.4.7 Systemic Oxidative Stress……………………………………………………… 111
4.4.8 Serum Inflammatory Markers……………………………………………….... 113

4.5 Discussion………………………………………………………………………. 113
4.5.1 Purple-fleshed Potato Reversed Colonic and Mesenteric Fat Oxidative Stress (GSH:GSSG Ratio) in HFD Fed Animals………………………………………. 114
4.5.2 Purple-fleshed Potato Reversed Colonic and Mesenteric Fat Inflammatory Markers in HFD Fed Animals……………………………………………………... 115
4.5.3 Purple-fleshed Potato Reversed Systemic Oxidative Stress Markers in HFD Fed Animals……………………………………………………………………………….. 117
4.5.4 Purple-fleshed Potato Suppressed Serum TNF-α in HFD Fed Animals…………... 119
4.5.5 Limitations of the Study……………………………………………………... 120

4.6 Conclusions…………………………………………………………………….. 122

4.7 References……………………………………………………………………… 123

CHAPTER V: PURPLE-FLESHED POTATOES PREVENTS HIGH-FAT DIET ELEVATED OXIDATIVE STRESS AND INNATE INFLAMMATION MARKERS IN A PIG MODEL………………………………………………………………… 133

5.1 Summary………………………………………………………………………... 133

5.2 Introduction…………………………………………………………………….. 134

5.3 Materials and Methods…………………………………………………………. 137
5.3.1 Animals……………………………………………………………………….. 137
5.3.2 Experimental Diets…………………………………………………………… 137
5.3.3 Blood, Urine and Tissue Collection………………………………………….. 141
5.3.4 Tissue Oxidative Stress………………………………………………………. 141
5.3.5 Real Time Polymerase Chain Reaction……………………………………… 141
5.3.6 Systemic Oxidative Stress Markers…………………………………………... 144
5.3.7 Serum Inflammatory Markers………………………………………………... 144
5.3.8 Statistical Analysis…………………………………………………………… 144

5.4 Results………………………………………………………………………….. 145
5.4.1 Physical Parameters………………………………………………………….. 145
5.4.2 Colonic GSH:GSSG Ratio…………………………………………………… 149
5.4.3 Colonic Inflammatory Markers……………………………………………… 150
5.4.4 Mesenteric Fat GSH:GSSG Ratio……………………………………………. 153
5.4.5 Mesenteric Fat Inflammatory Markers……………………………………… 154
5.4.6 Systemic Oxidative Stress…………………………………………………… 156
5.4.7 Serum Inflammatory Markers………………………………………………... 157

5.5 Discussion……………………………………………………………………… 161
5.5.1 Purple-Fleshed Potato Prevented Colonic and Mesenteric Fat Oxidative Stress (GSH:GSSG Ratio) in HFD Fed Animals………………………………………. 161
5.5.2 Potato (Purple and White-Fleshed) Prevented HFD Elevated Colonic and Mesenteric Fat Inflammatory Markers…………………………………………….. 162
5.5.3 Potato Diets Suppressed Systemic Oxidative Stress Markers in HFD Fed Animals………………………………………………………………………………... 164
5.5.4 Potato Diets Suppressed Serum TNF-α and IL-1β in HFD Fed Animals…………. 165

5.6 Conclusions…………………………………………………………………….. 167

5.7 References……………………………………………………………………… 169

CHAPTER VI: SUMMARY………………………………………………………………. 176

6.1 In vitro Study…………………………………………………………………... 176

6.2 Animal Study…………………………………………………………………... 177

APPENDICES (I – VII)……………………………………………………………. 179

LIST OF ABBREVIATIONS……………………………………………………… 188

LIST OF TABLES

Table 3.1: Combination index (CI) analysis of HCT-116 p53 +/+ and p53 -/- cells treated with different combinations of resveratrol and grape seed extract.

Table 4.1: Composition of diets used in the reversal study.

Table 4.2: Primers used in the manuscript for real time PCR in the reversal study.

Table 4.3: Phenolic and anthocyanin composition of potato chips by UPLC/MS.

Table 4.4: Growth performances (body weight, weight gain and feed intake) of pigs consuming experimental diets during the 5 week feeding period in the reversal study.

Table 5.1: Composition of diets used in the prevention study.

Table 5.2: Primers used in the manuscript for real time PCR in the prevention study.

Table 5.3: Growth performances (body weight, weight gain and feed intake) of pigs in the prevention study.

LIST OF FIGURES

Figure 2.1 Experiment design for the prevention and the reversal studies involving high-fat diet fed pig model.

Figure 3.1: Representative single ion UPLC-MS chromatograms of catechin and epicatechin monomers and their oligomers, and their gallate derivatives present in the grape seed extract used in the study.

Figure 3.2: Anti-proliferative, cytotoxic and apoptotic effects of resveratrol and grape seed extract.

Figure 3.3: Effect of combination of resveratrol and grape seed extract on cell cycle progression of HCT 116 colon cancer cells.

Figure 3.4: Synergistic combination of resveratrol and grape seed extract induced apoptosis in HCT-116 human colon cancer cell lines.

Figure 3.5: Apoptotic induction by the synergistic combination of resveratrol and grape seed extract involves pathways downstream of p53.

Figure 3.6: N-acetyl cysteine suppressed resveratrol-grape seed extract combination's anti-proliferative and pro-apoptotic properties.

Figure 3.7: Summary of the signaling pathways for resveratrol-grape seed extract combination-induced apoptosis.

Figure 4.1A: Typical total ion chromatograms from LC/MS analysis of purple- and white-fleshed potato phenolic extracts.

Figure 4.1B: Volcano plot of the features (mass by charge ratio/retention time) obtained after comparison of the UPLC/MS metabolite profile between purple and white-fleshed potato varieties.

Figure 4.2: Effect of potato supplemented high-fat diets on glutathione (GSH-reduced and GSSG-oxidized) status as measured using UPLC/PDA in the distal colon in the reversal study.

Figure 4.3A: Effect of potato supplemented high-fat diets on the relative expression of TLR-4 and NF-κB as measured using real time PCR in the distal colon mucosa in the reversal study.

Figure 4.3B: Effect of potato supplemented high-fat diets on the relative expression of TNF-α and COX-2 as measured using real time PCR in the distal colon mucosa in the reversal study.

Figure 4.3C: Effect of potato supplemented high-fat diets on the relative expression of TLR-2 as measured using real time PCR in the distal colon mucosa in the reversal study.

Figure 4.3D: Effect of potato supplemented high-fat diets on the relative expression of IL-10 and TGF-β as measured using real time PCR in the distal colon mucosa in the reversal study.

Figure 4.4: Effect of potato supplemented high-fat diets on the glutathione (GSH-reduced and GSSG-oxidized) status in the mesenteric fat measured using UPLC/PDA in the reversal study.

Figure 4.5A: Effect of potato supplemented high-fat diets on the relative expression of TNF-α and adiponectin as measured using real time PCR in the mesenteric fat in the reversal study.

Figure 4.5B: Effect of potato supplemented high-fat diets on the relative expression of NF-κB and TLR-4 as measured using real time PCR in the mesenteric fat in the reversal study.

Figure 4.5C: Effect of potato supplemented high-fat diets on the relative expression of IL-10 and TGF-β as measured using real time PCR in the mesenteric fat in the reversal study.

Figure 4.6: Effect of potato supplemented high-fat diets on serum levels of 8-isoprostane, malondialdehyde and TNF-α in the reversal study.

Figure 5.1: Effect of potato supplemented high-fat diets on the glutathione (GSH-reduced and GSSG-oxidized) status measured in the distal colon mucosa using UPLC/PDA in the prevention study.

Figure 5.2A: Effect of potato supplemented high-fat diets on the relative expression of TLR-4, NF-κB and TLR-2 as measured using real time PCR in the distal colon mucosa in the prevention study.

Figure 5.2B: Effect of potato supplemented high-fat diets on the relative expression of TNF-α and COX-2 as measured using real time PCR in the distal colon mucosa in the prevention study.

Figure 5.3: Effect of potato supplemented high-fat diets on the relative expression of IL-10 and TGF-β as measured using real time PCR in the distal colon mucosa in the prevention study.

Figure 5.4: Effect of potato supplemented high-fat diets on glutathione (GSH-reduced and GSSG-oxidized) status measured in the mesenteric fat using UPLC/PDA in the prevention study.

Figure 5.5A: Effect of potato supplemented high-fat diets on the relative expression of NF-κB and TLR-4 as measured using real time PCR in the mesenteric fat in the prevention study.

Figure 5.5B: Effect of potato supplemented high-fat diets on the relative expression of TNF-α and adiponectin as measured using real time PCR in the mesenteric fat in the prevention study.

Figure 5.6: Effect of potato supplemented high-fat diets on the relative expression of IL-10 and TGF-β as measured using real time PCR in the mesenteric fat in the prevention study.

Figure 5.7A: Effect of potato supplemented high-fat diets on urinary 8-isoprostane and 8-OHDG concentrations in the prevention study.

Figure 5.7B: Effect of potato supplemented high-fat diets on serum levels of TNF-α and IL-1β and fasting serum insulin and glucose in the prevention study.

Figure 5.7C Effect of potato supplemented high-fat diets on the serum lipid profile (Total Cholesterol, HDL-direct, LDL and Triglycerides) in the prevention study.

CHAPTER I

INTRODUCTION

Although some dietary fat is necessary for health, in countries like the US, the major issue is consuming too much rather than too little. It is commonly pointed out that people in the US have reduced their percentage of calories from fat over the last three decades; however, actual fat consumption (i.e., grams of fat per day) has remained quite high since the 1960s. The reduction in fat percentage is due to the fact that total calorie intake has increased rather than that fat intake has decreased (for men, average energy intake increased from 2,450 kcals to 2,618 kcals, and for women, from 1,542 kcals to 1,877 kcals (CDC 2004)). Excess dietary fat consumption is a concern because considerable research suggests that higher dietary fat consumption can lead to oxidative stress, elevated innate inflammatory response, and increased risk for chronic disease such as colon cancer (Reddy 2002, Lovejoy 2010, Silviera, Smith et al. 2012). Dietary modification can help reduce risk for such conditions. Increased consumption of bioactive compounds present in fruits and vegetables are inversely correlated with chronic disease risk (Hooper and Cassidy 2006, Pan, Lai et al. 2009). Plant bioactive compounds such as anthocyanins, proanthocyanidins, and stilbenoids like resveratrol (RSV) have shown to possess anti-inflammatory and anti-cancer properties in multiple *in vitro* and *in vivo* studies (Kowalczyk, Krzesinski et al. 2003, Cooke, Schwarz et al. 2006, Kaur, Singh et al. 2006, Vanamala, Reddivari et al. 2010). Grapes (proanthocyanidins and RSV), and purple-fleshed potatoes (anthocyanins and phenolic acids) are attractive delivery vehicles for these bioactive compounds as they are one of the highest consumed fruit and vegetable crops around the world.

RSV and grape seed extract (GSE, rich in proanthocyanidins, typically lacks RSV) have demonstrated anti-cancer properties in both *in vitro* and *in vivo* models. They have shown to alter p53 related pathways in suppression of colon cancer cell proliferation and induction of apoptosis typically at high concentrations difficult to achieve with dietary supplementation (Agarwal, Singh et al. 2002, Laurent, Besancon et al. 2004, Kaur, Singh et al. 2006, Kaur, Mandair et al. 2008, Vanamala, Reddivari et al. 2010, Vanamala, Radhakrishnan et al. 2011). Single bioactive compound approach has often failed during clinical trials to suppress colon cancer growth (Saldanha and Tollefsbol 2012). Therefore, there is a growing interest in using combination of bioactive compounds that act synergistically and at low concentrations to suppress cancer growth. There is no direct evidence linked to the possible synergistic or additive effect (and mechanism of action) of the RSV-GSE combination in human colon cancer cell line HCT-116 kinetics, especially in the presence of IGF-1, a mitogen elevated during obese conditions (Vanamala, Reddivari et al. 2010).

The potato is the third largest source of phenolic compounds in the human diet after oranges and apples (Chun, Kim et al. 2005). Purple-fleshed potatoes are rich in anthocyanins and phenolic acids with anti-oxidant and anti-inflammatory properties. Unlike other anthocyanin-rich sources such as blueberries, purple-fleshed potatoes are relatively inexpensive and widely available, but they are almost always consumed in processed form (Madiwale 2012). Thus, it is important to understand the effect of processing on the anti-inflammatory and anti-oxidant properties of purple-fleshed potato bioactive compounds *in vivo* before we present it as a functional food – a delivery vehicle for health promoting bioactive compounds.

The overall hypothesis we aimed to verify is that bioactive compounds from grapes and purple-fleshed potatoes (fresh, baked and chips) suppress colon cancer and HFD elevated

oxidative stress and inflammatory biomarkers, respectively. This hypothesis was tested using *in vitro* (human HCT-116 colon cancer cells) and *in vivo* (pig) models, respectively.

1.1 Aims

Aim 1: Determine if RSV and GSE synergistically suppress human HCT-116 colon cancer cell proliferation and induce apoptosis and determine the mechanisms of action of the combination.

Approach: Human colon cancer cells (HCT-116 p53 +/+ and p53 -/-) and normal colon epithelial cells (CRL-1831) treated with bioactive compounds from grapes (RSV, GSE) at different concentrations alone or in combination were assayed for their anti-proliferative and pro-apoptotic effects. We used inhibitor assays and western blotting technique to elucidate mechanisms of action of the RSV-GSE combination. Efficacy of RSV-GSE combination in presence of IGF-1 was also determined.

Outcomes: This *in vitro* study determines **1)** whether addition of RSV to proanthocyanidin-rich GSE, which lacks RSV, potentiates the chemopreventive properties of GSE; **2)** whether the RSV-GSE combination induces apoptosis and arrests the cell proliferation selectively in cancer cells not in the normal human colon epithelial cells; **3)** if the RSV-GSE combination works even in the presence of IGF-1 and **4)** molecular mechanisms underlying anti-cancer properties of the grape compounds in combination. These *in vitro* data will facilitate future *in vivo* studies to determine the anti-cancer properties of grape bioactive compounds using laboratory animal models.

Aim 2: Determine extent to which purple-fleshed potato consumption, even after processing, prevents or reverses HFD elevated oxidative stress, and innate inflammation markers in a pig model.

Approach: We performed two studies – a prevention study where 64 pigs, 3 weeks post-weaning, consumed one of the eight diets (n = 8): standard diet (SD), HFD and HFD supplemented with raw, baked or chipped purple or white-fleshed potatoes (10 % w/w) for 13 weeks; and a reversal study where pigs (12 weeks on HFD after 3 week post weaning period) consumed HFD containing 10/20 % purple or white-fleshed potato chips for additional 5 weeks (n = 8). Samples (distal colon mucosa, mesenteric fat, serum, urine) were assayed for markers of oxidative stress and inflammation. The pig with nutrient metabolism and gut structure anatomically and physiologically similar to humans was used in the study (Houpt, Houpt et al. 1979).

Outcomes: Elevated oxidative stress and inflammation markers in the colon, mesenteric fat and systemic circulation are central to progression of various chronic diseases. This study provided first *in vivo* evidence that delivery of anti-inflammatory and anti-oxidant bioactive compounds using purple-fleshed potatoes may potentially reduce risk for chronic diseases such as colonic inflammation and colon cancer.

1.2 References

Agarwal, C., R. P. Singh and R. Agarwal (2002). "Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release." Carcinogenesis **23**(11): 1869-1876.

CDC. (2004). "Trends in Intake of Energy and Macronutrients --- United States, 1971--2000." Morbidity and Mortality Weekly Report, from http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5304a3.htm.

Chun, O., D. Kim, N. Smith, D. Schroeder, J. Han and C. Lee (2005). "Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet." J Sci Food Agric **85**(10): 1715-1724.

Cooke, D., M. Schwarz, D. Boocock, P. Winterhalter, W. P. Steward, A. J. Gescher and T. H. Marczylo (2006). "Effect of cyanidin-3-glucoside and an anthocyanin mixture from bilberry on adenoma development in the ApcMin mouse model of intestinal carcinogenesis--relationship with tissue anthocyanin levels." Int J Cancer **119**(9): 2213-2220.

Hooper, L. and A. Cassidy (2006). "A review of the health care potential of bioactive compounds." J Sci Food Agric **86**(12): 1805-1813.

Houpt, K. A., T. R. Houpt and W. G. Pond (1979). "The pig as a model for the study of obesity and of control of food intake: a review." Yale J Biol Med **52**(3): 307-329.

Kaur, M., R. Mandair, R. Agarwal and C. Agarwal (2008). "Grape seed extract induces cell cycle arrest and apoptosis in human colon carcinoma cells." Nutr Cancer **60 Suppl 1**: 2-11.

Kaur, M., R. P. Singh, M. Gu, R. Agarwal and C. Agarwal (2006). "Grape seed extract inhibits in vitro and in vivo growth of human colorectal carcinoma cells." Clin Cancer Res **12**(20 Pt 1): 6194-6202.

Kowalczyk, E., P. Krzesinski, M. Kura, B. Szmigiel and J. Blaszczyk (2003). "Anthocyanins in medicine." Pol J Pharmacol **55**(5): 699-702.

Laurent, C., P. Besancon, C. Auger, J. M. Rouanet and B. Caporiccio (2004). "Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells." J Agric Food Chem **52**(11): 3301-3308.

Lovejoy, J. C. (2010). Fat: The Good, the Bad, and the Ugly. Nutrition Guide for Physicians. T. Wilson, G. A. Bray, N. J. Temple and M. B. Struble, Springer, 2009**:** 1-12.

Madiwale, G. (2012). Effect of genotype, storage and processing on the polyphenolic content, composition, in vitro anti-cancer activity and sensory attributes of colored-flesh potatoes. Master of Science Thesis, Colorado State University.

Pan, M. H., C. S. Lai, S. Dushenkov and C. T. Ho (2009). "Modulation of inflammatory genes by natural dietary bioactive compounds." J Agric Food Chem **57**(11): 4467-4477.

Reddy, B. S. (2002). "Types and amount of dietary fat and colon cancer risk: Prevention by omega-3 fatty acid-rich diets." Env Health Prev Med **7**(3): 95-102.

Saldanha, S. N. and T. O. Tollefsbol (2012). "The Role of Nutraceuticals in Chemoprevention and Chemotherapy and Their Clinical Outcomes." J Oncol **2012**(Article ID 192464).

Silviera, M. L., B. P. Smith, J. Powell and C. Sapienza (2012). "Epigenetic differences in normal colon mucosa of cancer patients suggest altered dietary metabolic pathways." Cancer Prev Res **5**(3): 374-384.

Vanamala, J., S. Radhakrishnan, L. Reddivari, V. B. Bhat and A. Ptitsyn (2011). "Resveratrol suppresses human colon cancer cell proliferation and induces apoptosis via targeting the pentose phosphate and the talin-FAK signaling pathways-A proteomic approach." Proteome Sci **9**(1): 49.

Vanamala, J., L. Reddivari, S. Radhakrishnan and C. Tarver (2010). "Resveratrol suppresses IGF-1 induced human colon cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways." BMC Cancer **10**: 238.

CHAPTER II

REVIEW OF LITERATURE

PART 1 – SYNERGY OF BIOACTIVE COMPOUNDS AGAINST COLON CANCER

2.1 General Overview of Bioactive Compounds

Bioactive compounds are secondary metabolites of plants that target physiological or cellular pathways and provide a beneficial health effect. Bioactive compounds typically occur in small amounts in foods, however, it is estimated that almost 0.2–1 g/day of these compounds are consumed as part of a regular healthy diet (Kris-Etherton, Hecker et al. 2002, Kris-Etherton, Lefevre et al. 2004). They belong to different classes and include phenolic acids, anthocyanins, carotenoids, stilbenes, glucosinolates, etc. They are found in fruits, vegetables, nuts, herbs etc., and in commonly consumed beverages such as wine, tea, and coffee. These compounds have demonstrated anti-oxidant and anti-inflammatory properties via targeting enzymes, cell surface and intracellular receptors, and by influencing gene expression, among other actions (Kris-Etherton, Hecker et al. 2002, Kris-Etherton, Lefevre et al. 2004). There has been wide interest in the field of bioactive compounds as they have been linked to a reduced risk for a variety of chronic diseases including cancer (Kris-Etherton, Hecker et al. 2002, Kris-Etherton, Lefevre et al. 2004). Moreover, they fit the characteristics of an ideal chemopreventive agent, as these compounds are selective to cancerous or precancerous cells, target most types of cancers, can be consumed orally, and is generally accepted by humans (Kaur, Agarwal et al. 2009).

2.2 Colon Cancer

Colon cancer is the 3rd most prevalent cancer in both men and women, and accounts for almost 9 % of total deaths due to all cancers. Colon cancer is highly affected by environmental factors including diet and lifestyle. It is a concerning fact that the highest incidence rates of colon cancer are observed in developed nations including the US (Marshall 2008). Studies dealing with the migration of individuals from developing nations (colon cancer risk is low) to developed countries have seen that within one generation, risk for colon cancer in these individuals is elevated (Marshall 2008). In addition, colon cancer rates are rapidly rising in developing nations and could be due to adoption of several features of the western lifestyle (Marshall 2008). Western dietary and lifestyle factors are linked to high incidence of colorectal cancer in developed countries. Diets rich in red and processed meat, refined starches, sugar, and saturated and trans-fatty acids but poor in fruits, vegetables, fiber, omega-3 fatty acids and whole grains are closely associated with an increased risk of colon cancer (Bruce, Wolever et al. 2000, Marshall 2008, Cappellani, Zanghi et al. 2013).

However, these factors are highly controllable and thus, colon cancer is highly preventable. Nutritional recommendations from the American Cancer Society include adequate intake of fruits and vegetable in a regular diet (Society 2012). A meta-analysis of case-control studies suggests that fruit and vegetable consumption in general is associated with a slight decrease in the risk of colon cancer (Marshall 2008). For example, fruit consumption was associated with a 13% decrease in colon cancer risk, and vegetable consumption was associated with a 40% decrease (Marshall 2008). Thus, the benefits from consuming a diet rich in fruit and vegetables could be attributed to the plethora of bioactive compounds present in them.

2.3 Resveratrol

Resveratrol (*trans*-3, 5, 4` trihydroxystilbene, RSV) is a polyphenol found in grapes and red wine among other plant products (Vanamala, Reddivari et al. 2010). RSV is synthesized in plant species in response to stress by the enzyme trihydroxystilbene synthase. Fresh grape skins contain 50-100 mg RSV per gram, and the concentration in wine ranges from 0.2 mg/l to 7.7 mg/l (Osei-Mensah 2011). The epidemiological finding of an inverse relationship between consumption of red wine and incidence of cardiovascular disease has been called the "French paradox" and the benefits of red wine consumption have been attributed to RSV (Vanamala, Tarver et al. 2008, Vanamala, Reddivari et al. 2010). RSV has demonstrated anti-oxidant, anti-inflammatory, anti-aging properties in a variety of studies (Baxter 2008). RSV interferes with all three stages of carcinogenesis: initiation, promotion and progression (Jang, Cai et al. 1997). We previously reported that RSV (> 100 μM) suppressed HT-29 human colon cancer cell proliferation and elevated apoptosis even in the presence of IGF-1 via suppression of IGF-1R/Akt/Wnt signaling pathways and activation of p53 (Vanamala, Reddivari et al. 2010). In other studies, RSV (100 μM) induced apoptosis independently of p53 in HCT-116 human colon cancer cells via mitochondrial intrinsic apoptotic pathway (Mahyar-Roemer, Katsen et al. 2001). In Caco-2 human colon cancer cells, RSV (> 100 μM) inhibited growth and proliferation, induced caspase-3 activation and apoptosis, and induced cell cycle arrest via modulation of cyclins and cylin dependent kinases (Wolter, Akoglu et al. 2001).

RSV elicited anti-cancer effects in animal and human models (Bishayee 2009). Oral resveratrol (200 μg/kg/day in drinking water) suppressed the number and multiplicity of azoxymethane-induced aberrant crypt foci, a precursor lesion for colon cancer, in the colon of rats. These effects were linked to alterations in Bax and p21 expression (Tessitore, Davit et al.

2000). In another rat study, RSV (8 mg/kg body weight) markedly reduced the number of 1,2-dimethylhydrazine induced aberrant crypt foci and incidence and size of tumors, possibly through the modulation of antioxidant defense status and activities of carcinogen-detoxifying enzymes (Sengottuvelan and Nalini 2006, Sengottuvelan, Senthilkumar et al. 2006, Sengottuvelan, Viswanathan et al. 2006). In a human study, daily oral doses of RSV at 0.5 or 1.0 g reduced colon tumor cell proliferation by 5 % (Patel, Brown et al. 2010). Anti-cancer properties of RSV in a variety of *in vitro* and *in vivo* models are reviewed elsewhere (Athar, Back et al. 2007).

2.4 Grape Seed Extract

Grape seed extract (GSE, lacks RSV, (Radhakrishnan, Reddivari et al. 2011)) is a bioactive mixture that is commonly consumed as a dietary supplement and is sold in the form of capsules or tablets (100–500 mg). The consumer interest in GSE has been primarily due to the high content of antioxidants in the form of proanthocyanidins in this extract. The antioxidant capacity of GSE is greater than known antioxidants such as vitamin C and E (Kaur, Agarwal et al. 2009). Grape seed proanthocyanidins have demonstrated a wide spectrum of biological, pharmacological, therapeutic, and chemopreventive properties in multiple models (Kaur, Agarwal et al. 2009).

Chemopreventive potential of GSE against colorectal cancer was evaluated *in vitro* in LoVo and HT-29 human colon cancer cell lines. GSE (> 50 µg/ml) suppressed the growth of these cancer cells. More importantly, GSE inhibited the growth of HT-29 cells *in vitro* as well as in tumor xenografts in athymic nude mice at dose of 200 mg/kg (Kaur, Singh et al. 2006). In animal models for colon cancer chemoprevention, grape seed proanthocyanidins (1 % w/w)

significantly inhibited azoxymethane-induced colonic aberrant crypt foci in a rat dual-organ tumor model (Singletary and Meline 2001). GSE (0.25 – 0.5 % w/w) prevented azoxymethane-induced colonic aberrant crypt foci formation in Fischer 344 rats and this was most likely via targeting β-catenin and NF-κB signaling (Velmurugan, Singh et al. 2010). Grape seed extract is generally well tolerated when taken by mouth. It has been used safely for up to 8 weeks in clinical trials. Human studies on anti-cancer efficacy of GSE are however, currently lacking (Medicine 2012). Anti-cancer properties of GSE and their mechanisms of action are summarized in detail elsewhere (Kaur, Agarwal et al. 2009).

2.5 Synergy of Bioactive Compounds

Consumption of fruits and vegetables has generally been associated with a decrease in cancer incidence and cardiovascular disease. As a result, numerous bioactive compounds have been isolated and identified, and their potential health-promoting effects have been evaluated extensively, both *in vitro* and *in vivo* (Kris-Etherton, Hecker et al. 2002, Kris-Etherton, Lefevre et al. 2004, Marshall 2008, Chuang and McIntosh 2011). However, purified phytochemicals may not necessarily exert the same beneficial health effect compared to when the compound is in its food matrix and among other bioactive compounds. There is a growing body of evidence that the actions of phytochemicals administered as dietary supplements alone do not explain the observed health benefits of diets rich in fruits, vegetables and whole grains (de Kok, van Breda et al. 2008). Relatively high doses of single bioactive agents may show potent anti-carcinogenic effects, however, the synergistic interactions between different dietary ingredients that potentiate the activities of any single constituent better explain the observed benefits of whole foods and diets in many epidemiological studies (Liu 2004, de Kok, van Breda et al. 2008). In a recent

study that compared GSE induced anti-cancer effects to the effects of its individual components, the researchers found that GSE was more potent in growth inhibition compared to its individual constituents epigallocatechin, procyanidins and their association (Dinicola, Cucina et al. 2012). Thus, the beneficial effect of fruit/vegetable consumption may not be the result of a single bioactive compound, but may arise from combination of different compounds.

In addition, bioactive compounds may have pleiotropic effects that in combination reduce the risk of chronic disease. Different compounds might target different pathways and the net effect might be a greater suppression of cancer cell growth (Kris-Etherton, Lefevre et al. 2004, Majumdar, Banerjee et al. 2009). Over the last few years, there have been many studies on bioactive components and their synergistic anti-cancer effects (Mertens-Talcott, Talcott et al. 2003, Aggarwal, Bhardwaj et al. 2004, Mertens-Talcott and Percival 2005, Vanamala, Glagolenko et al. 2008, Yang and Liu 2009, Chuang and McIntosh 2011, Del Follo-Martinez, Banerjee et al. 2013). However, there is no direct evidence linked to the possible synergistic or additive effect (and mechanism of action) of the grape bioactive components (RSV and GSE) in a colon cancer model.

2.6 Aim 1

Determine if RSV and GSE Synergistically Suppress Human HCT-116 Colon Cancer Cell Proliferation and Induce Apoptosis and Determine the Mechanisms of Action of the Combination.

Approach: Human colon cancer cells (HCT-116 p53 +/+ and p53 -/-) and normal colon epithelial cells (CRL-1831) treated with bioactive compounds from grapes – RSV and GSE at different concentrations alone or in combination were assayed for proliferation, apoptosis and p53-dependent pathways. These *in vitro* data will facilitate future *in vivo* studies to determine the anti-cancer properties of grape bioactive compounds using laboratory animal models.

Chemotherapeutic combination approaches have been used to reduce drug toxicity and obtain greater efficacy than the use of a single active component (Yang and Liu 2009). In a similar fashion, the phytochemicals in fruits could be used in combination as anti-cancer agents. However, there is little or no evidence for synergistic, additive, or antagonistic effects of grape compounds in combination against human colon cancer cells. This project provides evidence for the *in vitro* anti-cancer actions of the RSV-GSE combination which is already marketed as a popular supplement. *In vivo* confirmation of these results is clearly warranted.

PART 2 – POTATO BIOACTIVE COMPOUNDS AGAINST HIGH-FAT DIET ELEVATED OXIDATIVE STRESS AND INNATE INFLAMMATION

2.7 Diet and Oxidative Stress/Inflammation

Diets high in calories, especially from sugars, saturated fatty acids, and long-chain ω6 polyunsaturated fatty acids, and sedentary lifestyles contribute significantly to chronic diseases such as colon cancer (Chuang and McIntosh 2011). Life expectancy is predicted to decline in the US in the next few decades primarily due to chronic diseases now developing at early age, attributed mainly to poor diet and lifestyle (CDC 2009, CDC 2010).

Colonic health is especially a major problem in countries like the US where inflammatory conditions, such as inflammatory bowel disease (IBD) are prevalent. The prevalence in the US of IBD, including Crohns disease (CD) and ulcerative colitis (UC), is greater than 200 cases per 100,000, with the total number of IBD patients between 1 and 1.5 million (Rubin, Shaker et al. 2012). Chronic colonic inflammation observed in UC or CD patients is a risk factor for colon carcinogenesis (Terzić, Grivennikov et al. 2010, Ullman and Itzkowitz 2011). Epidemiologic evidence suggests that high-fat diet (HFD) consumption is a risk factor for the development of colonic disorders, including IBD and colon cancer (Vanamala, Tarver et al. 2008, Hou, Abraham et al. 2011). Chronic consumption of a HFD enhances colonic oxidative stress and innate inflammatory (Ding, Chi et al. 2010, Kim, Gu et al. 2012) response in rats (Slavin 2013) and humans (Sakamoto, Kono et al. 2005, Higgins 2014). Ingestion of HFD has shown to induce changes in gut bacteria, alter gut permeability, leading to elevated lipopolysaccharide (LPS, a component of gram negative bacteria) levels and toll-like receptor (TLR-4, receptor for LPS) activation in the colon (Kim, Gu et al. 2012).

Recent papers have reported that inflammation of colonic mucosa causes inflammatory responses in mesenteric fat (fat surrounding intestinal segments), a key component of visceral fat (Karagiannides, Kokkotou et al. 2006) and in liver and other organs (Li, Lelliott et al. 2008). In animal models with intracolonic trinitrobenzene sulfonic acid instillations (experimentally induced colitis), there was elevated expression of inflammatory cytokines in the mesenteric fat (Karagiannides, Kokkotou et al. 2006, Gambero, Marostica et al. 2007). HFD induced inflammation and altered gut permeability exposes gut microbiota and their products (e.g. Flagellin, LPS) to the mesenteric adipose tissue. LPS induced TLR-4/NF-κB innate inflammatory signaling has shown to promote macrophage (ATM) recruitment in the mesenteric fat depot. Although, other targets might be involved, current literature suggests that inflammation induced impaired gut barrier function and resulting leakage of microbial antigens induces inflammation in the mesenteric fat depot (Lam, Mitchell et al. 2011, Drouet, Dubuquoy et al. 2012, Kim, Gu et al. 2012).

Adipose tissue is no longer recognized as a passive storehouse of excess energy, but an active endocrine and secretory organ, and a major contributor to the elevated levels of a number of inflammatory proteins (Li, Lelliott et al. 2008). Adipose tissue inflammation is linked to systemic inflammation with elevated levels of circulating cytokines such as TNF-α, IL-1β, IL-6 etc. which are pro-inflammatory, and IL-10 and TGF-β which are anti-inflammatory (Li, Lelliott et al. 2008). Elevated low-grade systemic inflammation observed in animals on HFD has shown to be a silent killer as it forms the bedrock for development and progression of a variety of chronic inflammatory disorders such as rheumatoid arthritis, cancer etc. (Wilson, Finch et al. 2002, Li, Lelliott et al. 2008, Terzic, Grivennikov et al. 2010, Chuang and McIntosh 2011). HFD consuming animals have the colonic mucosa in a pro-inflammatory state, together with elevated

oxidative stress/inflammation in mesenteric fat and systemic circulation could contribute to elevated risk for chronic diseases (Li, Lelliott et al. 2008).

Oxidative stress plays a critical role in the pathogenesis of various diseases. Oxidative stress activates inflammatory pathways converging to either JNK activation or via the NF-κB pathways (Solinas and Karin 2010). The activated inflammatory cells further release reactive oxygen species (ROS) and reactive nitrogen species (Nagata 2005). The excessively produced ROS can injure cellular biomolecules such as nucleic acids, proteins, and lipids causing cellular and tissue damage, which can worsen state of inflammation. Chronic low grade oxidative stress and inflammation are commonly observed in HFD consuming models and are conducive to development of disorders such as colon cancer (Furukawa, Fujita et al. 2004, Gregersen, Samocha-Bonet et al. 2012). Indeed, Western-style diets induced oxidative stress and deregulated immune responses in the colon in a mouse model of sporadic colon cancer (Erdelyi, Levenkova et al. 2009).

Chronic inflammatory conditions have a long latency period and thus, there exists a window to institute appropriate dietary strategies and/or pharmacological interventions to prevent such disorders. There is a growing interest in finding natural plant-based strategies/agents against inflammatory disorders (Vanamala, Tarver et al. 2008). Dietary bioactive compounds (for e.g. anthocyanins, phenolic acids) can elicit pharmacological effects and can significantly alter activity of therapeutic agents by modulating biochemical pathways (Pan, Lai et al. 2009). Bioactive compounds like anthocyanins, phenolic acids and other polyphenols have shown potent anti-inflammatory properties in both animals and humans (Kim, Araki et al. 1998, Kowalczyk, Krzesinski et al. 2003, Baur and Sinclair 2006, Jurenka 2009, Chuang and McIntosh 2011). Anthocyanins, in particular, are known for their broad spectrum

action which include anti-inflammatory, anti-oxidant, anti-cancer properties etc. (Kowalczyk, Krzesinski et al. 2003).

2.8 The Potato

The potato (*Solanum tuberosum*) is indigenous to the central Andean region of South America and was introduced to Europe by the Spanish in the 16th century. Currently, the potato is the world's 4th largest food crop and the leading vegetable crop in the US, with per capita consumption of about 126 lbs annually (NPC 2005, 2006, NPC 2007). Potato is a good source of vitamins, minerals, and high quality proteins. The International Year of the Potato (2008) was officially launched at United Nations headquarters (New York) in October 2007 to focus world attention on the role potato can play in providing food security and eradicating poverty (UN 2008). This popularity makes potatoes and potato products an attractive "delivery system" for bioactive compounds in humans. However potatoes have been tarnished by bad press primarily due to being considered a high glycemic form of carbohydrate that could increase insulin resistance and risk of type 2 diabetes (Halton, Willett et al. 2006). It should be noted that studies providing the bad press are epidemiological and not experimental or clinical in nature.

Potatoes accumulate a wide variety of secondary metabolites including polyphenols. The polyphenol content of potato tubers ranges from 530-1770 µg/gfw (Reddivari 2007). Major tuber phenolic acids include caffeic acid, chlorogenic acid, ferulic acid, and cryptochlorogenic acid. Other phenolic acids in potato include neochlorogenic acid, p-coumaric acid, and ferulic acid amides (Reddivari 2007). Research has reported that chlorogenic acid constitutes up to 90 % of the total phenolic content of potato tubers (Friedman 1997). Chlorogenic acid, caffeic acid and p-coumaric acid have demonstrated anti-inflammatory properties in multiple studies (da Cunha,

Duma et al. 2004, Chao, Mong et al. 2010, Chauhan, Satti et al. 2011). Chlorogenic acid reduced liver inflammation and fibrosis through inhibition of TLR-4 signaling pathway in male Sprague-Dawley rats treated with carbon tetrachloride (CCl_4 – causes hepatotoxicity). Chlorogenic acid suppressed CCl_4 induced NF-κB activation, and hepatic mRNA expression and serum levels of TNF-α, IL-6 and IL-1β (Shi, Dong et al. 2013). Phenolics acids like caffeic acid have shown TNF-α lowering ability *in vivo* (Chao, Hsu et al. 2009). Caffeic acid and some of its derivatives such as caffeic acid phenethyl ester and octyl caffeate are potent antioxidants which present important anti-inflammatory actions (da Cunha, Duma et al. 2004).

Purple-fleshed potatoes are rich in phenolic acids and anthocyanins. We (Madiwale, Reddivari et al. 2011, Madiwale 2012, Madiwale, Reddivari et al. 2012) and others (Rodriguez-Saona, Giusti et al. 1998, Brown 2005) have shown that phenolic acids are present at amounts 5 – 12 times higher in purple-fleshed potatoes compared to their white-fleshed counterparts. Purple-fleshed cultivars had ~ 10 – 20 times greater anti-oxidant activity compared to white-fleshed potatoes and could be attributed mainly to the presence of anthocyanins and greater amounts of phenolic acids (Radhakrishnan, Reddivari et al. 2011, Madiwale, Reddivari et al. 2012). However, the anthocyanin content in these specialty cultivars greatly varies by variety of the potato. Purple-fleshed cultivars have shown to contain anthocyanins in a wide range (6-300 mg/100 gfw). The major anthocyanins identified were coumaryl–rutino–glucosides of petunidin, peonidin, malvidin and pelargonidin (Reddivari 2007).

There are numerous reports on the anti-inflammatory properties of anthocyanins both *in vitro* and *in vivo*. Anthocyanins from bilberries and black currants suppressed the secretion of pro-inflammatory cytokines such as IL-8, MCP-1, IL-1β, IL-6, and TNF-α in *in vitro* and animal models (Karlsen, Retterstol et al. 2007). The same anthocyanins (300 mg/d for 3 weeks)

inhibited NF-κB activation in monocytes and reduced plasma concentrations of pro-inflammatory mediators in healthy adults (Karlsen, Retterstol et al. 2007). Anthocyanin-rich berry extracts inhibited nitric oxide production in LPS/IFN-γ activated RAW 264.7 macrophages (Wang and Mazza 2002). In addition, anthocyanins from different sources (cherry, berries, soy) have demonstrated pronounced anti-inflammatory properties in multiple models (Rossi, Serraino et al. 2003, Tall, Seeram et al. 2004, Han, Sekikawa et al. 2006, Nizamutdinova, Kim et al. 2009). Reports demonstrate the anti-cancer properties of anthocyanins against colon cancer both *in vitro* and *in vivo* (Kang, Seeram et al. 2003, Zhao, Giusti et al. 2004, Lala, Malik et al. 2006).

However, potato is known to contain some undesirable compounds; which are either naturally occurring such as glycoalkaloids (Friedman 2006), or produced by heating during processing of potatoes at a high temperature like acrylamide. Glycoalkaloids found in the potato tubers include α-chaconine and α-solanine; which form approximately 95 % of the total glycoalkaloids. Glycoalkaloids are affected by pre-and postharvest factors such as poor growing conditions, sprouting, mechanical injury, fungal attack, exposure to light and sub-optimal storage conditions (Friedman 1997). However, most of freshly harvested commercial potato varieties contain safe levels of GAs, below the limit of 200 mg glycoalkaloids/kg of potato fresh weight (Knuthsen, Jensen et al. 2009).

Processing of potato such as frying and baking generate acrylamide which is formed from the reaction of reducing sugars (free glucose, fructose, sucrose and hydrolyzed starch during cold potato storage) with amino acid asparagine via Maillard reaction, which occurs during processing temperature above 212 °F (Pelucchi, La Vecchia et al. 2011). Acrylamide is considered as a neurotoxicant and carcinogen in animal models (Friedman and Levin 2008). Potato products such as French fries and potato chips contain a high level of acrylamide ranging

424-1739 µg/kg. Acrylamide however, is found in many foods specifically that have been roasted to temperatures above 120 ºC (for e.g. cocoa powder and chocolate, formed during cacao bean roasting). Current knowledge suggests that it is difficult to consume doses high enough to reach toxic levels or cause cancer. However, over time, it may be possible to consume enough (depending on diet) to cause enough DNA damage to lead to cancer (FDA 2013).

The potato is known for its carbohydrate content of which starch form a predominant percentage. A small but significant portion of this starch is resistant to digestion by enzymes in the stomach and small intestine, and so reaches the large intestine essentially intact where it is fermented by the gut bacteria to short chain fatty acids predominantly butyrate (Fung, Cosgrove et al. 2012, Haenen, Souza da Silva et al. 2013). This resistant starch is considered to have similar physiological effects and health benefits as fiber (Slavin 2013, Higgins 2014). It provides bulk, offers protection against colon cancer, improves glucose tolerance and insulin sensitivity, improves lipid profile etc. (Dias 2012). However, the amount of resistant starch in potatoes depends much on preparation methods. Cooking and then cooling potatoes significantly increases resistant starch. For example, cooked potato starch contains about 7% resistant starch, which increases to about 13% upon cooling (Englyst, Kingman et al. 1992).

The potato is an important nutrient-dense component of the Western diet and represents a potentially very important source of energy for combating hunger around the world. Color-fleshed potatoes offer similar profile of compounds as traditional white-fleshed potatoes, in addition they contain bioactive compounds such as anthocyanins and carotenoids (Brown 2005, Camire, Kubow et al. 2009).

2.9 Potato Processing

It is well known that processing changes the physical and chemical composition of foods (Spanos, Wrolstad et al. 1990, Price, Bacon et al. 1997), thus affecting their bioactivity (Nicoli, Anese et al. 1999, Dewanto, Wu et al. 2002). Potatoes are almost always consumed after processing (baked, chipped, fried, boiled or microwaved) making it critical to understand the effect of such processing techniques on the activity of bioactive compounds in potatoes.

Reports from our lab suggest that when compared with raw samples, depending on the potato variety, baking decreased or increased the phenolic content (Madiwale, Reddivari et al. 2012). Purple-fleshed clones CO97215-2P/P and CO97227-2P/PW showed increased phenolic content post-baking, whereas phenolic content decreased in white- and yellow-fleshed cultivars (Madiwale, Reddivari et al. 2012). Researchers have reported both an increase and a decrease in the phenolic content post-baking (Im, Suh et al. 2008, Xu, Li et al. 2009, Blessington, Nzaramba et al. 2010, Navarre, Shakya et al. 2010). The increase in the phenolic content post-baking could be due to release of bound phenolic compounds during baking. Cooking may weaken the matrix thus, improving the extractability of phenolic compounds and inactivate enzymes that use phenolic compounds as substrate (Ezekiel, Singh et al. 2011). However, effects of processing cannot be generalized for all potato clones as results differ depending on potato genotype – this could be due to the differential content of free vs bound phenolic compounds in different varieties. Thus, both genotype and processing affect the bioactive content of potatoes (Madiwale 2012). Among different processing methods, chipping/slicing increases the surface area, thus there is greater degradation of bioactive compounds compared to baked and raw samples (Madiwale 2012, Madiwale, Reddivari et al. 2012).

Processing of potatoes also affects the content and composition of other bioactive compounds in the potato including vitamin C, glycoalkaloids, acrylamide and resistant starch. Effects of processing on the potato toxicants (glycoalkaloids and acrylamide) and resistant starch are reviewed in the earlier section. *In vitro* data from our lab showed that ethanolic extracts of raw, baked and chipped purple-fleshed potatoes suppressed proliferation and elevated apoptosis in HCT-116 human colon cancer cell line. Anti-proliferative and pro-apoptotic properties of baked potatoes were similar to that of raw potatoes, while chipping caused a significant reduction in the biological activity (Madiwale 2012). However, there is a dearth of data on health benefits of potato bioactive compounds, especially after processing *in vivo*. It is important to document, using appropriate *in vivo* models, if processed (raw vs. baked vs. chips) anthocyanin-rich purple-fleshed potatoes retain their anti-oxidant and anti-inflammatory properties *in vivo*, as this aspect has received little consideration.

2.10 Pig as a Model

There are major drawbacks to the use of rodents or cats/dogs as models for the study of human ingestive behavior. In addition to the anatomical and physiological differences between rodents and humans, rats and mice were originally granivores. Dogs and cats are carnivores whose natural diet and meal patterns differ from those of humans. The expense and scarcity of primates have limited their use in studies on food intake (Houpt, Houpt et al. 1979). Thus, the pig is an excellent model for studies of nutrition and food intake in humans. An ideal model of the human gastrointestinal tract should faithfully replicate the ecology of the gut. Such a model would be susceptible to diseases affecting humans, develop similar symptoms, mimic the adults or infant gastrointestinal tract and would be experimentally tractable and affordable. The pig

model used in the study fulfills several criteria; specifically it is experimentally tractable and it is a clinically relevant model of the human gastrointestinal tract (Houpt, Houpt et al. 1979, Cooper, Berry et al. 1997, Pond and Lei 2000).

2.11 Aim 2

Determine Extent to Which Purple-Fleshed Potato Consumption, Even After Processing, Prevents or Reverses HFD Elevated Oxidative Stress, and Inflammation Markers in a Pig Model.

Approach: We performed two studies – a prevention study where 64 pigs, 3 weeks post-weaning, consumed one of the eight diets: standard diet (SD), HFD and HFD supplemented with raw, baked or chipped purple or white-fleshed potatoes (10 % w/w) for 13 weeks; and a reversal study where pigs (12 weeks on HFD after 3 weeks post weaning) consumed HFD containing 10 or 20 % purple or white-fleshed potato chips for additional 5 weeks (n = 8). Experiment design is presented in Figure 2.1. Distal colon mucosa, mesenteric fat, serum, and urine were assayed for oxidative stress and inflammatory markers. The pig with nutrient metabolism and gut structure anatomically and physiologically similar to humans than any other non-primate mammal was an appropriate model used in the study.

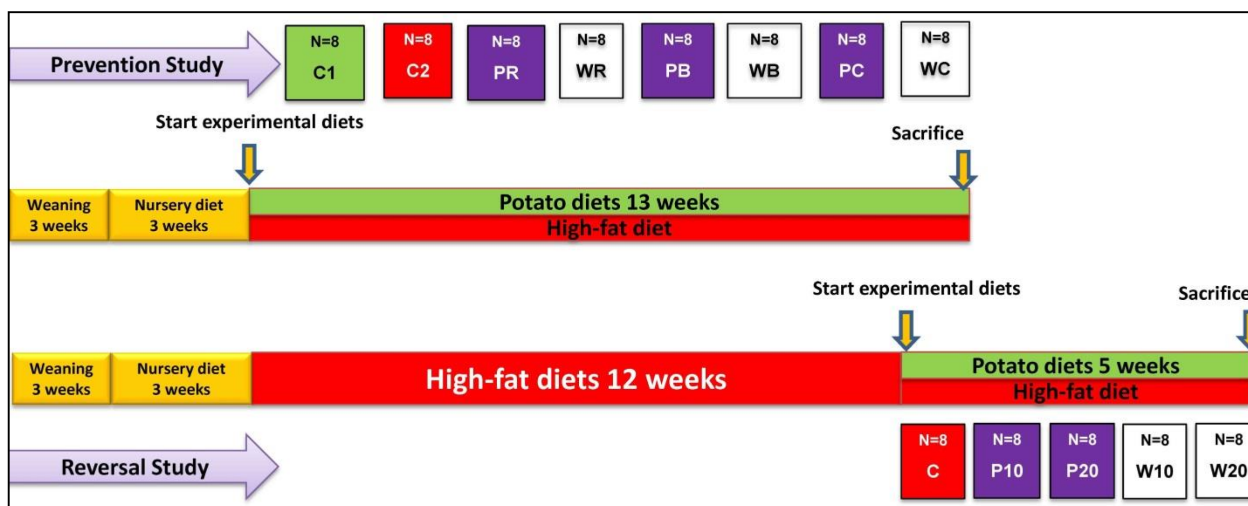

Figure 2.1 Experiment design for the prevention and the reversal studies involving high-fat diet consuming pig model. C1, standard control, C & C2, high-fat diet control in reversal and prevention studies, respectively, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively in the prevention study at 10 % supplementation. The numbers 10 and 20 in the reversal study experimental diets indicate 10 and 20 % potato supplementation in the diet (chips, w/w).

Providing answers to the *in vivo* stability and bioactivity of phenolic acids, anthocyanins and other bioactive compounds in the purple-fleshed potato, post processing will assist producers and consumers to make a more informed decision on shifting production to color-fleshed potatoes and determine purchasing preferences, respectively. In addition, understanding the anti-oxidant and anti-inflammatory effects of potato consumption in a human relevant pig model will provide information to help overcome the negative attitudes held by consumers regarding potato consumption.

2.11 References

(2006). Potato statistical yearbook, National Potato Council (NPC).

Aggarwal, B. B., A. Bhardwaj, R. S. Aggarwal, N. P. Seeram, S. Shishodia and Y. Takada (2004). "Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies." Anticancer Res **24**(5A): 2783-2840.

Athar, M., J. H. Back, X. Tang, K. H. Kim, L. Kopelovich, D. R. Bickers and A. L. Kim (2007). "Resveratrol: a review of preclinical studies for human cancer prevention." Toxicol App Pharmacol **224**(3): 274-283.

Baur, J. A. and D. A. Sinclair (2006). "Therapeutic potential of resveratrol: the in vivo evidence." Nat Rev Drug Discov **5**(6): 493-506.

Baxter, R. A. (2008). "Anti-aging properties of resveratrol: review and report of a potent new antioxidant skin care formulation." J Cosmetic Dermatol **7**(1): 2-7.

Bishayee, A. (2009). "Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials." Cancer Prev Res **2**(5): 409-418.

Blessington, T., M. Nzaramba, D. Scheuring, A. Hale, L. Reddivari and J. Miller (2010). "Cooking methods and storage treatments of potato: Effects on carotenoids, antioxidant activity, and phenolics." Am J Potato Res **87**(6): 479-491.

Brown, C. R. (2005). "Antioxidants in potato." Am J Potato Res **82**: 163-172.

Bruce, W. R., T. M. Wolever and A. Giacca (2000). "Mechanisms linking diet and colorectal cancer: the possible role of insulin resistance." Nutr Cancer. **37**(1): 19-26.

Camire, M. E., S. Kubow and D. J. Donnelly (2009). "Potatoes and Human Health." Critical Rev Food Sci Nutr **49**(10): 823-840.

Cappellani, A., A. Zanghi, M. Di Vita, A. Cavallaro, G. Piccolo, P. Veroux, E. Lo Menzo, V. Cavallaro, P. de Paoli, M. Veroux and M. Berretta (2013). "Strong correlation between diet and development of colorectal cancer." Front Biosci **18**: 190-198.

CDC. (2009). "The Power of Prevention. Chronic diseases - the public health challenge of the 21st century " National Center for Chronic Disease Prevention and Health Promotion, from http://www.cdc.gov/chronicdisease/pdf/2009-power-of-prevention.pdf.

CDC (2010). "http://www.cdc.gov/obesity/data/trends.html."

Chao, C. Y., M. C. Mong, K. C. Chan and M. C. Yin (2010). "Anti-glycative and anti-inflammatory effects of caffeic acid and ellagic acid in kidney of diabetic mice." Mol Nutr Food Res **54**(3): 388-395.

Chao, P. C., C. C. Hsu and M. C. Yin (2009). "Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice." Nutr Metab **6**: 33.

Chauhan, P. S., N. K. Satti, V. K. Sharma, P. Dutt, K. A. Suri and S. Bani (2011). "Amelioration of inflammatory responses by Chlorogenic acid via suppression of pro-inflammatory mediators." J Applied Pharma Sci **1**(4): 67-75.

Chuang, C. C. and M. K. McIntosh (2011). "Potential mechanisms by which polyphenol-rich grapes prevent obesity-mediated inflammation and metabolic diseases." Annu Rev Nutr **31**: 155-176.

Cooper, D. A., D. A. Berry, V. A. Spendel, A. L. Kiorpes and J. C. Peters (1997). "The domestic pig as a model for evaluating olestra's nutritional effects." J Nutr **127**(8 Suppl): 1555S-1565S.

da Cunha, F. M., D. Duma, J. Assreuy, F. C. Buzzi, R. Niero, M. M. Campos and J. B. Calixto (2004). "Caffeic acid derivatives: in vitro and in vivo anti-inflammatory properties." Free Radical Res **38**(11): 1241-1253.

de Kok, T. M., S. G. van Breda and M. M. Manson (2008). "Mechanisms of combined action of different chemopreventive dietary compounds: a review." Euro J Nutr **47 Suppl 2**: 51-59.

Del Follo-Martinez, A., N. Banerjee, X. Li, S. Safe and S. Mertens-Talcott (2013). "Resveratrol and quercetin in combination have anticancer activity in colon cancer cells and repress oncogenic microRNA-27a." Nutr Cancer. **65**(3): 494-504.

Dewanto, V., X. Wu, K. Adom and R. Liu (2002). "Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity." J Agric Food Chem **50**(10): 3010-3014.

Dias, J. S. (2012). "Nutritional Quality and Health Benefits of Vegetables: A Review." Food Nutr Sci **3**(10): 1354-1374.

Ding, S., M. M. Chi, B. P. Scull, R. Rigby, N. M. Schwerbrock, S. Magness, C. Jobin and P. K. Lund (2010). "High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse." PLoS One **5**(8): e12191.

Dinicola, S., A. Cucina, A. Pasqualato, F. D'Anselmi, S. Proietti, E. Lisi, G. Pasqua, D. Antonacci and M. Bizzarri (2012). "Antiproliferative and Apoptotic Effects Triggered by Grape Seed Extract (GSE) versus Epigallocatechin and Procyanidins on Colon Cancer Cell Lines." Int J Mol Sci **13**(1): 651-664.

Drouet, M., L. Dubuquoy, P. Desreumaux and B. Bertin (2012). "Visceral fat and gut inflammation." Nutrition **28**(2): 113-117.

Englyst, H. N., S. M. Kingman and J. H. Cummings (1992). "Classification and measurement of nutritionally important starch fractions." Euro J Clin Nutr **46 Suppl 2**: S33-50.

Erdelyi, I., N. Levenkova, E. Y. Lin, J. T. Pinto, M. Lipkin, F. W. Quimby and P. R. Holt (2009). "Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer." J Nutr. **139**(11): 2072-2078.

Ezekiel, R., N. Singh, S. Sharma and A. Kaur (2013). "Beneficial phytochemicals in potato -- a review." Food Res Int **50**(2): 487–496.

FDA. (2013). "Acrylamide Questions and Answers." Foodborne Illness and Contaminants, from http://www.fda.gov/food/foodborneillnesscontaminants/chemicalcontaminants/ucm053569.htm.

Friedman, M. (1997). "Chemistry, Biochemistry, and Dietary Role of Potato Polyphenols. A Review." J Agric Food Chem **45**(5): 1523-1540.

Friedman, M. (2006). "Potato glycoalkaloids and metabolites: roles in the plant and in the diet." J Agric Food Chem **54**(23): 8655-8681.

Friedman, M. and C. E. Levin (2008). "Review of methods for the reduction of dietary content and toxicity of acrylamide." J Agric Food Chem **56**(15): 6113-6140.

Fung, K. Y., L. Cosgrove, T. Lockett, R. Head and D. L. Topping (2012). "A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate." Br J Nutr **108**(5): 820-831.

Furukawa, S., T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, O. Nakayama, M. Makishima, M. Matsuda and I. Shimomura (2004). "Increased oxidative stress in obesity and its impact on metabolic syndrome." J Clin Investigation **114**(12): 1752-1761.

Gambero, A., M. Marostica, M. J. Abdalla Saad and J. Pedrazzoli, Jr. (2007). "Mesenteric adipose tissue alterations resulting from experimental reactivated colitis." Inflammatory Bowel Dis **13**(11): 1357-1364.

Gregersen, S., D. Samocha-Bonet, L. K. Heilbronn and L. V. Campbell (2012). "Inflammatory and Oxidative Stress Responses to High-Carbohydrate and High-Fat Meals in Healthy Humans." J Nutr Metab **2012**: 1-8.

Haenen, D., C. Souza da Silva, J. Zhang, S. J. Koopmans, G. Bosch, J. Vervoort, W. J. Gerrits, B. Kemp, H. Smidt, M. Muller and G. J. Hooiveld (2013). "Resistant starch induces catabolic but suppresses immune and cell division pathways and changes the microbiome in the proximal colon of male pigs." J Nutr. **143**(12): 1889-1898.

Halton, T. L., W. C. Willett, S. Liu, J. E. Manson, M. J. Stampfer and F. B. Hu (2006). "Potato and french fry consumption and risk of type 2 diabetes in women." Am J Clin Nutr **83**(2): 284-290.

Han, K. H., M. Sekikawa, K. Shimada, M. Hashimoto, N. Hashimoto, T. Noda, H. Tanaka and M. Fukushima (2006). "Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats." Br J Nutr **96**(6): 1125-1133.

Higgins, J. A. (2014). "Resistant starch and energy balance: impact on weight loss and maintenance." Crit Rev Food Sci Nutr **54**(9): 1158-1166.

Hou, J. K., B. Abraham and H. El-Serag (2011). "Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature." Am J Gastroenterol **106**(4): 563-573.

Houpt, K. A., R. T. Houpt and W. G. Pond (1979). "The Pig as a Model for the Study of Obesity and of Control of Food Intake: A Review." Yale J Biol Med **52**: 307-329.

Im, H., B. Suh, S. Lee, N. Kozukue, M. Ohnisi-Kameyama, C. Levin and M. Friedman (2008). "Analysis of phenolic compounds by high-performance liquid chromatography and liquid chromatography/mass spectrometry in potato plant flowers, leaves, stems, and tubers and in home-processed potatoes." J Agric Food Chem **56**(9): 3341-3349.

Jang, M., L. Cai, G. O. Udeani, K. V. Slowing, C. F. Thomas, C. W. Beecher, H. H. Fong, N. R. Farnsworth, A. D. Kinghorn, R. G. Mehta, R. C. Moon and J. M. Pezzuto (1997). "Cancer chemopreventive activity of resveratrol, a natural product derived from grapes." Science **275**(5297): 218-220.

Jurenka, J. S. (2009). "Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research." Altern Med Rev **14**(2): 141-153.

Kang, S. Y., N. P. Seeram, M. G. Nair and L. D. Bourquin (2003). "Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells." Cancer Lett **194**(1): 13-19.

Karagiannides, I., E. Kokkotou, M. Tansky, T. Tchkonia, N. Giorgadze, M. O'Brien, S. E. Leeman, J. L. Kirkland and C. Pothoulakis (2006). "Induction of colitis causes inflammatory responses in fat depots: evidence for substance P pathways in human mesenteric preadipocytes." Proc Natl Acad Sci USA **103**(13): 5207-5212.

Karlsen, A., L. Retterstol, P. Laake, I. Paur, S. K. Bohn, L. Sandvik and R. Blomhoff (2007). "Anthocyanins inhibit nuclear factor-kappaB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults." J Nutr. **137**(8): 1951-1954.

Kaur, M., C. Agarwal and R. Agarwal (2009). "Anticancer and cancer chemopreventive potential of grape seed extract and other grape-based products." J Nutr. **139**(9): 1806S-1812S.

Kaur, M., R. P. Singh, M. Gu, R. Agarwal and C. Agarwal (2006). "Grape seed extract inhibits in vitro and in vivo growth of human colorectal carcinoma cells." Clin Canc Res **12**(20 Pt 1): 6194-6202.

Kim, J. M., S. Araki, D. J. Kim, C. B. Park, N. Takasuka, H. Baba-Toriyama, T. Ota, Z. Nir, F. Khachik, N. Shimidzu, Y. Tanaka, T. Osawa, T. Uraji, M. Murakoshi, H. Nishino and H. Tsuda (1998). "Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation." Carcinogenesis **19**(1): 81-85.

Kim, K. A., W. Gu, I. A. Lee, E. H. Joh and D. H. Kim (2012). "High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway." PLoS One **7**(10): e47713.

Knuthsen, P., U. Jensen, B. Schmidt and I. K. Larsen (2009). "Glycoalkaloids in potatoes: Content of glycoalkaloids in potatoes for consumption." J Food Comp analy **22**(6): 577-581.

Kowalczyk, E., P. Krzesinski, M. Kura, B. Szmigiel and J. Blaszczyk (2003). "Anthocyanins in medicine." Pol J Pharmacol **55**(5): 699-702.

Kris-Etherton, P. M., K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel and T. D. Etherton (2002). "Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer." Am J Med **113 Suppl 9B**: 71S-88S.

Kris-Etherton, P. M., M. Lefevre, G. R. Beecher, M. D. Gross, C. L. Keen and T. D. Etherton (2004). "Bioactive compounds in nutrition and health-research methodologies for

establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis." Ann Rev Nutr **24**: 511-538.

Lala, G., M. Malik, C. Zhao, J. He, Y. Kwon, M. M. Giusti and B. A. Magnuson (2006). "Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats." Nutr Cancer **54**(1): 84-93.

Lam, Y. Y., A. J. Mitchell, A. J. Holmes, G. S. Denyer, A. Gummesson, I. D. Caterson, N. H. Hunt and L. H. Storlien (2011). "Role of the gut in visceral fat inflammation and metabolic disorders." Obesity **19**(11): 2113-2120.

Li, H., C. Lelliott, P. Hakansson, K. Ploj, A. Tuneld, M. Verolin-Johansson, L. Benthem, B. Carlsson, L. Storlien and E. Michaelsson (2008). "Intestinal, adipose, and liver inflammation in diet-induced obese mice." Metabolism **57**(12): 1704-1710.

Liu, R. H. (2004). "Potential synergy of phytochemicals in cancer prevention: mechanism of action." J Nutr. **134**(12 Suppl): 3479S-3485S.

Madiwale, G. (2012). Effect of genotype, storage and processing on the polyphenolic content, composition, in vitro anti-cancer activity and sensory attributes of colored-flesh potatoes. Master of Science Thesis, Colorado State University.

Madiwale, G. P., L. Reddivari, D. G. Holm and J. Vanamala (2011). "Storage elevates phenolic content and antioxidant activity but suppresses antiproliferative and pro-apoptotic properties of colored-flesh potatoes against human colon cancer cell lines." J Agric Food Chem **59**(15): 8155-8166.

Madiwale, G. P., L. Reddivari, M. Stone, D. G. Holm and J. Vanamala (2012). "Combined effects of storage and processing on the bioactive compounds and pro-apoptotic

properties of color-fleshed potatoes in human colon cancer cells." J Agric Food Chem **60**(44): 11088-11096.

Mahyar-Roemer, M., A. Katsen, P. Mestres and K. Roemer (2001). "Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse." Int J Canc **94**(5): 615-622.

Majumdar, A. P., S. Banerjee, J. Nautiyal, B. B. Patel, V. Patel, J. Du, Y. Yu, A. A. Elliott, E. Levi and F. H. Sarkar (2009). "Curcumin synergizes with resveratrol to inhibit colon cancer." Nutr Cancer **61**(4): 544-553.

Marshall, J. R. (2008). "Prevention of colorectal cancer: diet, chemoprevention, and lifestyle." Gastroenterol Clin North America **37**(1): 73-82, vi.

Medicine, N. C. f. C. a. A. (2012). "Grape Seed Extract." Herbs at a Glance, from http://nccam.nih.gov/health/grapeseed/ataglance.htm.

Mertens-Talcott, S. U. and S. S. Percival (2005). "Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells." Cancer letters **218**(2): 141-151.

Mertens-Talcott, S. U., S. T. Talcott and S. S. Percival (2003). "Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells." J Nutr. **133**(8): 2669-2674.

Nagata, M. (2005). "Inflammatory cells and oxygen radicals." Curr Drug Targets Inflamm Allergy **4**(4): 503-504.

Navarre, D., R. Shakya, J. Holden and S. Kumar (2010). "The Effect of Different Cooking Methods on Phenolics and Vitamin C in Developmentally Young Potato Tubers." Am J Potato Res **87**(4): 350-359.

Nicoli, M., M. Anese and M. Parpinel (1999). "Influence of processing on the antioxidant properties of fruit and vegetables." Trends Food Sci Tech **10**(3): 94-100.

Nizamutdinova, I. T., Y. M. Kim, J. I. Chung, S. C. Shin, Y. K. Jeong, H. G. Seo, J. H. Lee, K. C. Chang and H. J. Kim (2009). "Anthocyanins from black soybean seed coats stimulate wound healing in fibroblasts and keratinocytes and prevent inflammation in endothelial cells." Food Chem Toxicol **47**(11): 2806-2812.

NPC (2005). Potato statistical yearbook, National Potato Council (NPC).

NPC (2007). Potato statistical yearbook, National Potato Council (NPC).

Osei-Mensah, M. (2011). Synthesis of Resveratrol Ester Derivatives Using Selective Enzymatic Hydrolysis. Master of Science Thesis, East Tennessee State University.

Pan, M. H., C. S. Lai, S. Dushenkov and C. T. Ho (2009). "Modulation of inflammatory genes by natural dietary bioactive compounds." J Agric Food Chem **57**(11): 4467-4477.

Patel, K. R., V. A. Brown, D. J. Jones, R. G. Britton, D. Hemingway, A. S. Miller, K. P. West, T. D. Booth, M. Perloff, J. A. Crowell, D. E. Brenner, W. P. Steward, A. J. Gescher and K. Brown (2010). "Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients." Cancer Res **70**(19): 7392-7399.

Pelucchi, C., C. La Vecchia, C. Bosetti, P. Boyle and P. Boffetta (2011). "Exposure to acrylamide and human cancer—a review and meta-analysis of epidemiologic studies." Annals Oncol **22**(7): 1487.

Pond, W. G. and X. G. Lei (2000). Of pigs and people. Swine Nutrition. Washington, D.C., CRC Press**:** 3-18.

Price, K., J. Bacon and M. Rhodes (1997). "Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*)." J Agric Food Chem **45**(3): 938-942.

Radhakrishnan, S., L. Reddivari, G. Madiwale, S. W. Kim and J. Vanamala (2011). "Purple potato anthocyanins exert anti-cancer properties and suppress oxidative stress even after processing." Potato Association of America - PAA 2011.

Radhakrishnan, S., L. Reddivari, R. Sclafani, U. N. Das and J. Vanamala (2011). "Resveratrol potentiates grape seed extract induced human colon cancer cell apoptosis." Front Biosci (Elite Ed) **3**: 1509-1523.

Reddivari, L. (2007). Influence of genetic variability on specialty potato functional components and their effect on prostate cancer cell lines. Doctor of Philosophy, Texas A&M Universtiy.

Rodriguez-Saona, L. E., M. M. Giusti and R. E. Wrolstad (1998). "Anthocyanin pigment composition of red-fleshed potatoes." J Food Sci **63**(3): 458-465.

Rossi, A., I. Serraino, P. Dugo, R. Di Paola, L. Mondello, T. Genovese, D. Morabito, G. Dugo, L. Sautebin, A. P. Caputi and S. Cuzzocrea (2003). "Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation." Free Radic Res **37**(8): 891-900.

Rubin, D. C., A. Shaker and M. S. Levin (2012). "Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer." Front Immunol **3**: 107.

Sakamoto, N., S. Kono, K. Wakai, Y. Fukuda, M. Satomi, T. Shimoyama, Y. Inaba, Y. Miyake, S. Sasaki, K. Okamoto, G. Kobashi, M. Washio, T. Yokoyama, C. Date and H. Tanaka

(2005). "Dietary risk factors for inflammatory bowel disease: a multicenter case-control study in Japan." Inflamm Bowel Dis **11**(2): 154-163.

Sengottuvelan, M. and N. Nalini (2006). "Dietary supplementation of resveratrol suppresses colonic tumour incidence in 1,2-dimethylhydrazine-treated rats by modulating biotransforming enzymes and aberrant crypt foci development." Br J Nutr **96**(1): 145-153.

Sengottuvelan, M., R. Senthilkumar and N. Nalini (2006). "Modulatory influence of dietary resveratrol during different phases of 1,2-dimethylhydrazine induced mucosal lipid-peroxidation, antioxidant status and aberrant crypt foci development in rat colon carcinogenesis." Biochim Biophys Acta **1760**(8): 1175-1183.

Sengottuvelan, M., P. Viswanathan and N. Nalini (2006). "Chemopreventive effect of trans-resveratrol--a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis." Carcinogenesis **27**(5): 1038-1046.

Shi, H., L. Dong, J. Jiang, J. Zhao, G. Zhao, X. Dang, X. Lu and M. Jia (2013). "Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway." Toxicol **303**: 107-114.

Singletary, K. W. and B. Meline (2001). "Effect of grape seed proanthocyanidins on colon aberrant crypts and breast tumors in a rat dual-organ tumor model." Nutr Cancer. **39**(2): 252-258.

Slavin, J. (2013). "Fiber and prebiotics: mechanisms and health benefits." Nutrients **5**(4): 1417-1435.

Society, A. C. (2012). "Diet and activity factors that affect risks for certain cancers." ACS Guidelines on Nutrition and Physical Activity for Cancer Prevention, from

http://www.cancer.org/healthy/eathealthygetactive/acsguidelinesonnutritionphysicalactivityforcancerprevention/acs-guidelines-on-nutrition-and-physical-activity-for-cancer-prevention-dietand-activity.

Solinas, G. and M. Karin (2010). "JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction." FASEB J **24**(8): 2596-2611.

Spanos, G., R. Wrolstad and D. Heatherbell (1990). "Influence of processing and storage on the phenolic composition of apple juice." J Agric Food Chem **38**(7): 1572-1579.

Tall, J. M., N. P. Seeram, C. Zhao, M. G. Nair, R. A. Meyer and S. N. Raja (2004). "Tart cherry anthocyanins suppress inflammation-induced pain behavior in rat." Behav Brain Res **153**(1): 181-188.

Terzic, J., S. Grivennikov, E. Karin and M. Karin (2010). "Inflammation and colon cancer." Gastroenterology **138**(6): 2101-2114 e2105.

Tessitore, L., A. Davit, I. Sarotto and G. Caderni (2000). "Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression." Carcinogenesis **21**(8): 1619-1622.

Ullman, T. A. and S. H. Itzkowitz (2011). "Intestinal Inflammation and Cancer." Gastroenterol **140**(6): 1807-1816.e1801.

UN. (2008). "International Year of the Potato 191 session 60." from http://www.undemocracy.com/A-RES-60-191/page_1/rect_230,647_688,680.

Vanamala, J., A. Glagolenko, P. Yang, R. J. Carroll, M. E. Murphy, R. A. Newman, J. R. Ford, L. A. Braby, R. S. Chapkin, N. D. Turner and J. R. Lupton (2008). "Dietary fish oil and pectin enhance colonocyte apoptosis in part through suppression of PPARdelta/PGE2 and elevation of PGE3." Carcinogenesis **29**(4): 790-796.

Vanamala, J., L. Reddivari, S. Radhakrishnan and C. Tarver (2010). "Resveratrol suppresses IGF-1 induced human colon cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways." BMC Cancer **10**: 238.

Vanamala, J., C. C. Tarver and P. S. Murano (2008). "Obesity-enhanced colon cancer: functional food compounds and their mechanisms of action." Curr Cancer Drug Targets **8**(7): 611-633.

Velmurugan, B., R. P. Singh, R. Agarwal and C. Agarwal (2010). "Dietary-feeding of grape seed extract prevents azoxymethane-induced colonic aberrant crypt foci formation in fischer 344 rats." Mol Carcinogenesis **49**(7): 641-652.

Wang, J. and G. Mazza (2002). "Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-gamma-activated RAW 264.7 macrophages." J Agric Food Chem **50**(4): 850-857.

Wilson, C. J., C. E. Finch and H. J. Cohen (2002). "Cytokines and cognition--the case for a head-to-toe inflammatory paradigm." J Am Geriatr Soc **50**(12): 2041-2056.

Wolter, F., B. Akoglu, A. Clausnitzer and J. Stein (2001). "Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines." J Nutr. **131**(8): 2197-2203.

Xu, X., W. Li, Z. Lu, T. Beta and A. Hydamaka (2009). "Phenolic content, composition, antioxidant activity, and their changes during domestic cooking of potatoes." J Agric Food Chem **57**(21): 10231-10238.

Yang, J. and R. H. Liu (2009). "Synergistic effect of apple extracts and quercetin 3-beta-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro." J Agric Food Chem **57**(18): 8581-8586.

Zhao, C., M. M. Giusti, M. Malik, M. P. Moyer and B. A. Magnuson (2004). "Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth." J Agric Food Chem **52**(20): 6122-6128.

CHAPTER III

RESVERATROL POTENTIATES GRAPE SEED EXTRACT INDUCED HUMAN COLON CANCER CELL APOPTOSIS VIA ACTIVATION OF P53-DEPENDENT SIGNALING PATHWAYS.

3.1 Summary

Colon cancer is the third leading cause of cancer deaths in men and women. We and others have reported grape seed extract (GSE) and resveratrol (RSV) are potent chemopreventive agents against colon cancer both *in vitro* and *in vivo,* however, these compounds work in the colon only at relatively high concentrations. Since, phytonutrients occur as complex mixtures in fruits and vegetables and may work together to modulate their cancer preventive/protective properties; we hypothesized that RSV and GSE may act in concert with each other in potentiating their anti-cancer properties *in vitro* at sub-optimal doses. In this study, we showed that sub-optimal doses of RSV (~25 μM) potentiated GSE (≤ 35 μg/ml) chemoprotective effects via suppressing colon cancer cell proliferation and elevating apoptosis via activation of p53 dependent pathways. Elevation of apoptosis was more pronounced in p53 +/+ cells compared to p53 -/- cells. Apoptosis strongly correlated with phosphorylated p53 (serine 15) levels and Bax:Bcl-2 ratio, key players in the mitochondrial apoptotic pathway. Inhibitors of caspase-3 and anti-oxidants (quenching free radicals) attenuated apoptosis induced by the combination. RSV-GSE combination suppressed proliferation and induced apoptosis of HCT-116 colon cancer cells in the presence of mitogenic growth factor IGF-1, but did not suppress proliferation or induce apoptosis of normal colonic epithelial cell line CRL-1831 demonstrating specificity to cancer

cells. These observations suggest that RSV-GSE combination and other such naturally occurring phytonutrients need to be studied and assessed in combination as they would occur in nature, rather than individually.

3.2 Introduction

In 2009, the American Cancer Society estimated there were more than 100,000 new cases and over 40,000 deaths occurred due to colon cancer alone in the US (American Cancer Society 2009). Since colon cancer has a long latency period before it is detected even in its pre-clinical stage (such as colon polyp) (Grady and Carethers 2008), an opportunity exists to institute appropriate dietary preventive strategies to halt its progression. Dietary bioactive phytochemicals with chemopreventive properties that occur in nature as complex mixtures in fruits, vegetables, grains and herbs, are particularly suited for this purpose (Liu 2004). However, little information is available on additive, synergistic or antagonistic interactions among these phytochemicals. Resveratrol (RSV), grape seed extract (GSE) and their combination(s) are marketed as popular dietary supplements. Independently, RSV and GSE showed anti-cancer activity both *in vitro* and *in vivo.* But, no studies have been performed or reported on their possible additive or synergistic chemoprevention/protective effects.

Vitis vinifera (Grape vine) is a rich source of several bioactive compounds including anthocyanins, flavonoids, polyphenols, proanthocyanidins, and stilbenes (Nassiri-Asl and Hosseinzadeh 2009). GSE, a mixture containing about 95% standardized proanthocyanidins, is a popular dietary supplement due to its anti-cancer and anti-inflammatory properties (Agarwal et al. 2002). *In vitro* studies showed that GSE has significant growth inhibitory action on a variety of colon cancer cells in a dose and time dependent manner (Kaur et al. 2008). GSE significantly

inhibited cell viability and elevated apoptosis in cancer cells without altering the viability of the normal colon cell lines, thus selectively targeting cancer cells (Laurent et al. 2004). GSE induced G_1 phase arrest and caspase-3 mediated apoptosis in cancer cells (Laurent et al. 2004; Sharma et al. 2004; Kaur et al. 2006). GSE might thus exert its beneficial effects by elevating apoptosis and suppressing proliferative pathways. Even though GSE is available through different vendors, researchers have reported that regardless of the commercial source, GSE can produce comparable biological effects via cell growth suppression in a panel of human colon cancer cell lines (Kaur et al. 2008).

It is well known that GSE typically lacks RSV, except in special preparations, where RSV is added back in (Khanna et al. 2001). RSV, a stilbenoid derived from the skin of red grapes, has been shown to be active against various cancers *in vitro* and *in vivo* (Asensi et al. 2002; Bhat and Pezzuto 2002; Sexton et al. 2006; Alkhalaf 2007). RSV interferes with all three stages of carcinogenesis: initiation, promotion and progression (Jang et al. 1997; Jang and Pezzuto 1999; Aziz et al. 2003). We have previously shown that RSV has anti-proliferative and pro-apoptotic properties against HT-29 and SW480 human colon cancer cell lines even in the presence of mitogenic insulin like growth factor-1 (IGF-1) by elevating phosphorylated p53 (ser 15) and suppressing IGF-1R/Wnt signaling (Vanamala et al. 2010). However, RSV was effective at relatively high concentrations in *in vitro* studies (RSV > 25-100 µM in different cell lines). Though in an occasional instance, RSV was found to be active at low concentrations *in vitro* (Hope et al. 2008). In view of these evidences, we opined that addition of RSV at relatively low concentrations (~ 25 µM) to a natural food supplement such as GSE might potentiate the effectiveness of GSE in suppressing colon cancer cell growth, as both of them exist in the fruit

matrix in the form of mixtures. Furthermore, the inhibitory efficacy of such a RSV-GSE combination on colon carcinogenesis has not yet been reported.

Insulin like growth factor-1 (IGF-1) is a growth factor and the IGF axis is frequently activated during obesity and thus, could play a critical role in obesity-promoted colon cancer (Ma et al. 1999; LeRoith and Roberts 2003; Wei et al. 2005). The IGF system includes ligands, receptors, and ligand-binding proteins (IGFBPs). Positive energy balance and chronic hyperinsulinemia observed in obese conditions may deregulate colonocyte growth kinetics, as elevated insulin and suppressed IGFBP-1 and IGFBP-2 levels increase the pool of free or bioavailable IGF-1. A larger concentration of bioavailable IGF-1 can activate the IGF-1 receptor (IGF-1R is over expressed in colon cancer cells), and stimulate colonocyte proliferation (LeRoith and Roberts 2003; Frystyk 2004; Davies et al. 2006; Ahmed et al. 2007; Vanamala et al. 2008). Previously, we showed that RSV suppressed IGF-1R protein levels *in vitro* in human colon cancer cells (Vanamala et al. 2010).

Chemotherapeutic combination approaches have been used to reduce drug toxicity and obtain greater efficacy compared to a single active component (Yang and Liu 2009). In a similar fashion, the phytochemicals in fruits could be used in combination as anti-cancer agents. However, there is little or no evidence for synergistic, additive, or antagonistic effects of grape compounds in combination against human colon cancer cells. We hypothesized that RSV and GSE work synergistically to suppress colon cancer cell proliferation, at least, in part by inducing apoptosis via p53 dependent mechanisms. P53, a critical tumor suppressor gene, is frequently deregulated in many cancers and is activated upon DNA damage (Galluzzi et al. 2008). Even though GSE induced apoptosis in a p53 dependent manner in skin cancer fibroblasts (Roy et al. 2005), information on GSE's p53 dependent effects on colon cancer cells is limited. Results of

the present study suggest that RSV potentiates the anti-proliferative and pro-apoptotic properties of GSE in a p53-dependent manner. However, this combination induced apoptosis in p53 -/- cells, but to a lesser extent. Apoptosis was elevated via p53 activation, and Bax/Bcl-2/ROS and caspase-3 pathways. Even in the presence of IGF-1, a mitogenic growth factor elevated during obesity, RSV-GSE combination suppressed proliferation and induced apoptosis in HCT-116 p53 +/+ cells. The promising *in vitro* results of the RSV-GSE combination in inducing apoptosis of colon cancer cells in a synergistic manner, via p53-dependent pathways, even in the presence of IGF-1, implies that this combination has therapeutic potential and warrants further investigation using *in vivo* studies.

3.3 Materials and Methods

3.3.1 Chemicals

Grape seed extract (GSE, ORAC value 9000-13000 μmole Trolox equivalents/g, total phenolic content > 85% gallic acid equivalents was a generous gift from San Joaquin Valley Concentrates (Fresno, CA). It is important to note that GSE has been shown to produce comparable biological actions irrespective of its commercial source (Kaur et al. 2008). GSE is a mixture of mainly dimers, trimers and other oligomers of catechin and epicatechin and their gallate derivatives. UPLC-MS analysis of GSE used in the study detected presence of catechin and epicatechin monomers and their oligomers, and their gallate derivatives indicating that GSE had a similar profile as used in earlier studies ((Agarwal et al. 2007; Weber et al. 2007), Figure 3.1). We know that GSE typically lacks resveratrol (RSV) and UPLC-MS analysis of GSE confirmed the absence of RSV. We could not detect RSV, indicating RSV content in GSE is at

best below 0.016% by mass (value obtained by standard curve) even if we assume 90% ionization suppression due to the complex matrix of GSE.

Fetal bovine serum (FBS) was purchased from Fisher Scientific (Pittsburgh, PA). McCoy's 5A medium (modified, 1X) was purchased from Invitrogen (Carlsbad, CA). The p53 inhibitor Pifithrin-α, the caspase-8 inhibitor z-IETD-FMK, and the caspase-3 inhibitor z-DEVD-FMK were purchased from Calbiochem (San Diego, CA). N-acetylcysteine, catalase enzyme, Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12), 5-florouracil (5-FU) and RSV were from the Sigma Chemical Co. (St. Louis, MO). IGF-1 was obtained from the R&D Systems (Minneapolis, MN).

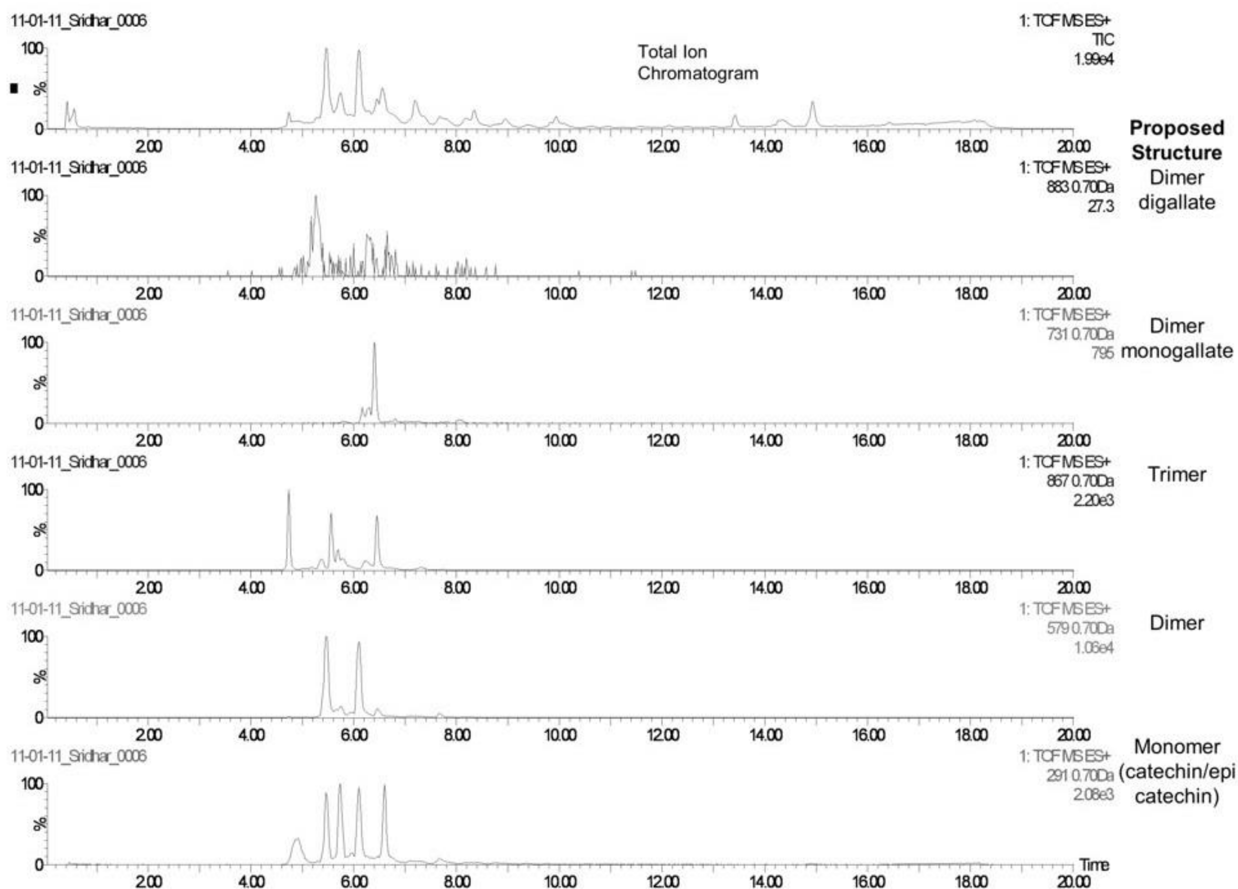

Figure 3.1 Representative single ion UPLC-MS chromatograms of catechin and epicatechin monomers and their oligomers, and their gallate derivatives present in the grape seed extract used in the study prepared at 5 mg/ml in water-methanol mixture. Detailed procedure for LC/MS is mentioned in the materials and methods.

3.3.2 LC-MS Analysis of Grape Seed Extract

GSE was dissolved in water-methanol mixture to attain a concentration of 5 mg/ml. 1 µl injections of the solution were performed on a Waters Acquity UPLC system. Separation was performed using a Waters Acquity UPLC HSS T3 column (1.8 µM, 1.0 x 100 mm), using a gradient from solvent A (Water + 0.1% formic acid) to solvent B (95% methanol, 5% water, 0.1% formic acid). Injections were made in 100% A, which was held for 2 minutes, there was a

13 minute linear gradient to 100% B which was held at 100% B for 2 minutes then returned to starting conditions over 0.1 minute, and finally allowed to reequilibrate for 2.9 minutes. Flow rate was constant at 140 µl/min for the duration of the run. The column was held at 50°C, samples were held at 5°C. Column eluent was infused into a Micromass Q-Tof Micro MS fitted with an electrospray source. Data was collected in positive ion mode, scanning from 50-1200 at a rate of 0.9 scans per second with 0.1 second interscan delay. Calibration was performed prior to sample analysis via infusion of sodium formate solution, with mass accuracy within 5 ppm. The capillary voltage was held at 2200V, the source temp at 130°C, and the desolvation temperature at 300°C at a nitrogen desolvation gas flow rate of 400 l/hr. The quadrupole was held at collision energy of 7 volts.

3.3.3 Cell Lines

The HCT-116 p53 +/+ and p53 -/- colon cancer cell lines were obtained from Dr. Bert Vogelstein (School of Medicine, the Johns Hopkins University, Baltimore, MD, USA). Cells were maintained at 37°C with 5% CO_2 and grown in McCoy's 5A medium supplemented with L-glutamine, 25 mM HEPES buffer, 100 ml/l fetal bovine serum and 10 ml/L Penicillin-Streptomycin mix. CRL-1831 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained at 37°C with 5% CO_2 and grown in Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12) supplemented with 2.2 g/l sodium bicarbonate, 0.2 g/l bovine serum albumin, 100 ml/l fetal bovine serum and 10 ml/l Penicillin-Streptomycin mix. Cell cultures at approximately 75% confluence were used for all experimental treatments.

3.3.4 Cell Proliferation Assay

Cell viability was assessed by a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (WST-1 assay; Roche Diagnostics, Indianapolis, IN) following the supplier's protocol. WST-1 assay was used after validating the assay using cell counting. Briefly, cells were grown in 96-well plates (5,000 cells/well), overnight at 37°C, in a 5% CO_2 incubator and treated with RSV and/or GSE for indicated time period. After addition of WST-1 reagent (1:10 dilution in media), cells were incubated for 3 h and absorbance at 450 nm was measured by a micro plate reader. The experiments were repeated three times each time in triplicate and data were expressed as the means ± SEs. For experiments using IGF-1, cell proliferation was measured via cell counting using Cellometer (Nexcelom Biosciences, Lawrence, MA). The experiments were repeated three times each time in triplicate and data were expressed as the means ± SEs.

3.3.5 Cytotoxicity Detection

To validate, if the concentrations of RSV and GSE used in the study are not toxic to the cells (induce necrosis), a dose response cytotoxicity detection assay using different concentrations of RSV and GSE was done. HCT-116 p53 +/+ and p53 -/- cells (5,000/well) were seeded in 96-well plates and after treatment for 24 h, the Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) was used to measure the Lactate-dehydrogenase (LDH) activity in supernatants of cells treated with respective treatments, according to the instruction manual protocol. Briefly, after 30 min incubation at 37°C, absorbance was recorded on a micro plate reader at 492 nm. The experiments were done in triplicate and repeated at least three times, and data were expressed as the means ± SE. Triton X-100 was the positive control. Cytotoxicity was

calculated with the formula: % Cytotoxicity = (experimental value - low control) X 100 / (high control - low control), where low control is the assay medium plus cells and high control is the assay medium plus 2 % Triton X-100 plus cells.

3.3.6 Apoptosis Assay

Apoptosis induction was measured using 2 different assays; a nucleosomal fragmentation assay (Cell Death Detection Enzyme Linked ImmunoSorbent Assay (ELISA), Roche Diagnostics, Indianapolis, IN) followed by confirmation using caspase-3 cleavage (Caspase-Glo 3/7 assay, Promega, Madison, WI). Briefly, for the nucleosome ELISA assay, 100,000 cells were plated per well in 12-well plates, and then treated with the respective treatments. After 12 h or 24 h incubation, cells were counted and assayed for apoptosis as per the manufacturer's protocol. Absorbance was recorded on a micro plate reader at 405 nm. The optical density was normalized to total number of cells in each treatment. For the caspase-glo 3/7 assay, cells were incubated for 12 h or 24 h with respective treatments. After incubation, cells were trypsinized and approximately 20,000 cells from each treatment were incubated with 100 µl of caspase-glo 3/7 reagent for 3 h. The luminescence of each sample was measured using a luminometer. DMSO was the solvent control. These experiments were repeated at least twice and each time in triplicate and data were expressed as the means ± SEs.

3.3.7 Experimental Design of Combination Study

A two-way combination of RSV and GSE against HCT-116 p53 +/+ and p53 -/- cell proliferation was designed (Yang and Liu 2009). The IC 50 values of RSV and GSE were determined on the basis of the dose-response curve (Figure 3.2A and 3.2B). Based on IC 50

values of RSV and GSE, a series of concentrations were used to select a few combinations that might demonstrate synergistic, additive or antagonistic response (C1-C9, Table 3.1).

The combination effects were analyzed by the combination index (CI) method. The CI is based on the classic isobologram equation (Sharma et al. 2004); $CI = D1/d1 + D2/d2$; where D1 and D2 are the doses of RSV and GSE, respectively, in the combination system; d1 and d2 are the doses of RSV and GSE alone for the same fractional inhibition, respectively. For data analysis of combinations, $CI \leq 1$, $CI = 1$, and $CI > 1$ indicate synergistic, additive, and antagonistic effects, respectively (Sharma et al. 2004; Yang and Liu 2009). C1 and C2 were chosen for further experiments based on low concentrations of RSV (~ 25 µM) and its ability to induce apoptosis comparable to high doses of RSV and GSE (100 µM or 100 µg/ml) in HCT-116 p53 +/+ colon cancer cells (Table 3.1).

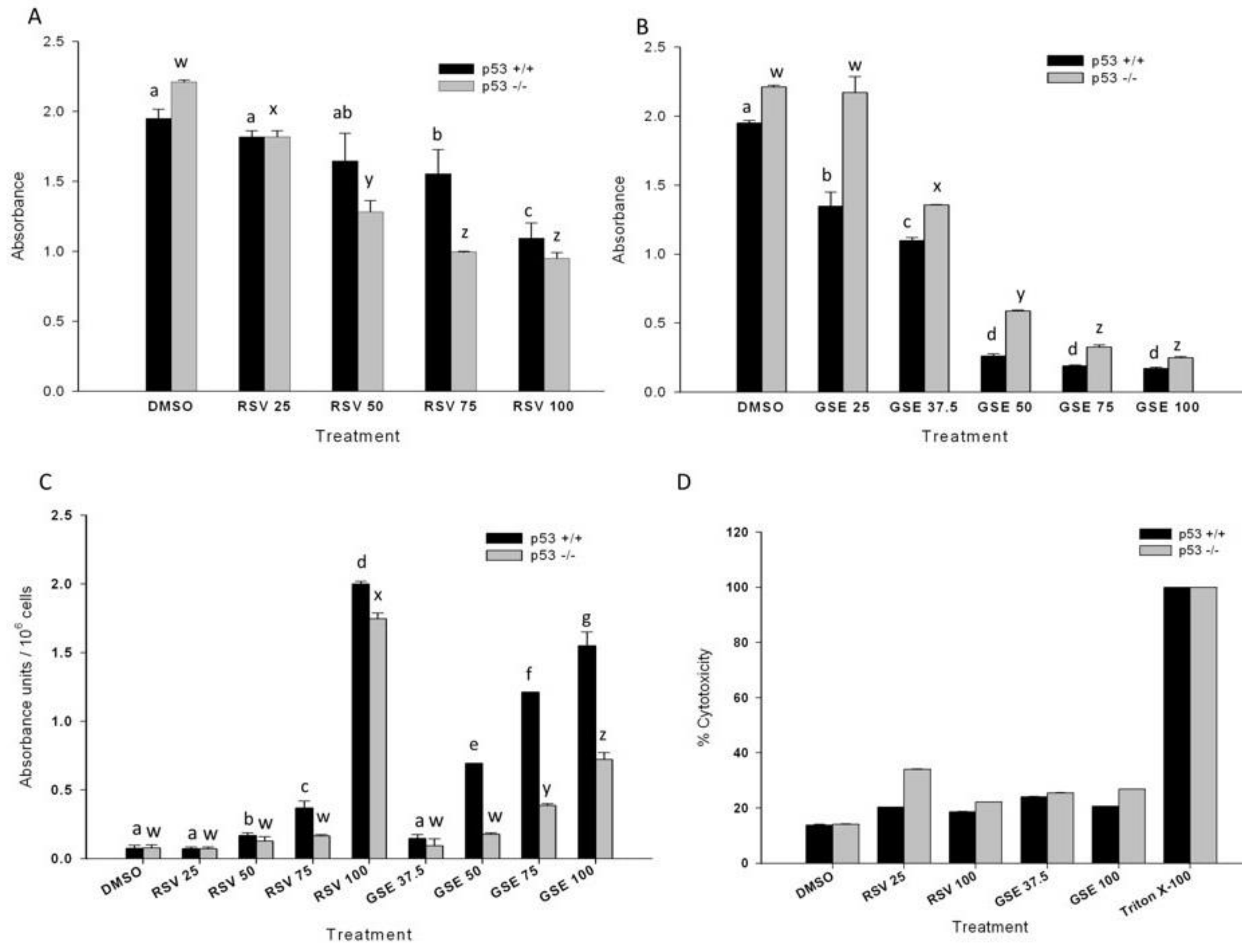

Figure 3.2 Anti-proliferative, cytotoxic and apoptotic effects of resveratrol (RSV) and grape seed extract (GSE). (A) RSV and (B) GSE suppressed HCT-116 colon cancer cell proliferation and (C) induced apoptosis but do not show any (D) cytotoxic effects. HCT-116 p53 +/+ and -/- cells were treated with RSV (25, 50, 75, and 100 μM) or GSE (25, 37.5, 50, 75 and 100 μg/ml) for 48 h and cell proliferation was determined using WST-1 assay and cytotoxicity was detected in the supernatants using LDH assay. HCT-116 p53 +/+ and -/- cells were treated with RSV (25, 50, 75 and 100 μM) or GSE (37.5, 50, 75 and 100 μg/ml) for 12 h and induction of apoptosis was determined by cell death detection ELISA assay. The rate of apoptosis was expressed as absorbance value measured at 405 nm. Results were expressed as mean ± SE for three replicate experiments for each treatment group. DMSO served as a solvent control. Triton X-100 was used

as a positive control for LDH assay. Means that differ by a common letter (a, b, c, d, e, f, g for p53 +/+ cells and w, x, y, z for p53 -/- cells) differ ($P \leq 0.05$).

Table 3.1 Combination index (CI) analysis of HCT-116 p53 +/+ and p53 -/- cells treated with different combinations of resveratrol (RSV) and grape seed extract (GSE). HCT-116 p53 +/+ and -/- cells were treated with combination of GSE and RSV at different concentrations for 48 h and cell proliferation was determined using WST-1 assay and apoptosis (24 h, only in p53 +/+ cells) was measured using caspase-glo 3/7 assay. Fractional inhibition was calculated as a percentage with respect to control and CI values were calculated based on method of Chou and Talalay (Chou and Talalay 1983). C1 and C2 (in bold) were chosen for further experiments based on low concentrations of RSV (~ 25 µM) and its ability to induce apoptosis comparable to high doses of RSV and GSE (100 µM or 100 µg/ml) in HCT-116 p53 +/+ colon cancer cells.

Treatment			**P53 +/+ cells**			**P53 -/- cells**	
Combinations	**RSV (µM)**	**GSE (µg/ml)**	**Fractional inhibition (%)**	**CI value**	**Apoptosis (X 100 Lumens)**	**Fractional inhibition (%)**	**CI value**
C1	**27**	**35.2**	**70.17**	**0.668**	**201.7**	**74.16**	**0.687**
C2	**23.59**	**50.93**	**79.03**	**0.812**	**245.0**	**75.17**	**0.858**
C3	15.73	33.95	70.05	0.561	129.4	72.47	0.608
C4	23.6	30	59.82	0.61	139.7	59.95	0.792
C5	31.45	67.9	81.64	1.078	383.4	80.36	1.008
C6	31.5	40	54.79	0.846	187.8	68.63	0.884

C7	41.25	24.75	48.24	0.755	99.1	66.86	0.753
C8	45	58.75	83.93	1.09	317.0	77.43	1.067
C9	55	33	63.11	0.896	192.0	72.57	0.893

3.3.8 Fluorescence Activated Cell Sorting Analysis (FACS)

HCT-116 p53 +/+ and p53 -/- cells were plated at a density of 15×10^6 cells per 100 mm plate and after 24 h treatment with either the control or the RSV-GSE combinations, cells were trypsinized and centrifuged. The pellet was resuspended with 1 ml of PI staining buffer containing 4 mM sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide and 200 μg/ml RNAse and incubated for 10 minutes at 37°C in the dark, and the final concentration of sodium chloride was adjusted to 0.15 M. Cells were analyzed using MoFlo (Dako Colorado, Inc.) flow cytometer and high speed cell sorter. Results were reported as percent cells in each phase of the cell cycle.

3.3.9 Western Blot Analysis

HCT-116 p53 +/+ and p53 -/- cells were seeded at a density of 1.5×10^5 cells/ml in DMEM F-12 media with 5% charcoal-stripped fetal bovine serum for 24 h. After treating cells for 24 h, protein was extracted into a high-salt buffer containing 1% protease and phosphatase inhibitor cocktail from Thermo Scientific (Rockford, IL), and protein concentrations were determined by using the BCA Protein Assay kit from Thermo Scientific (Rockford, IL). Cell lysates (45 μg) were incubated at 98°C for 5 minutes and separated by 10% polyacrylamide gels running at 120 V for 2 h in 1X running buffer, and electrophoretically transferred to Immobilon-FL, low fluorescent PVDF membranes from Millipore (Billerica, MA) at 30 V for 98 min in Tris-glycine transfer buffer. The membranes were blocked with Superblock blocking buffer from Thermo Scientific (Rockford, IL) for 2 h at room temperature. The membranes were incubated with rabbit polyclonal anti-pp53 antibody, rabbit polyclonal anti-Bax antibody, both from Cell Signaling (1:1,000; Danvers, MA), and rabbit polyclonal anti-Bcl-2, goat polyclonal anti-β-actin

both from Santa Cruz Biotechnology (1:500 for Bcl-2, 1: 5000 for β-actin; Santa Cruz, CA) for 2 h at room temperature. Membranes were subsequently probed with anti-goat or anti-rabbit IR Dye secondary antibodies, Licor Biosciences (all 1:10,000, Lincoln, NE). Membranes were scanned and quantified with Odyssey infrared imaging system, Licor Biosciences (Lincoln, NE) using Odyssey software (Licor Biosciences). Membranes were also probed with respective IgG-HRP secondary antibodies from Santa Cruz Biotechnology (1:20,000; Santa Cruz, CA) and scanned using UVP imaging software (UVP BioDoc-It® Imaging System; Upland, CA). β-actin served as a loading control.

3.3.10 IGF-1 Treatment

Cells were plated at a density of 5 X 10^4 per well in 12-well plates in DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum. After 24 h, cells were treated with DMSO (solvent control), 25 nM IGF-1 and/or combinations of RSV and GSE (C1 and C2), and anti-proliferative properties of the RSV-GSE combinations were evaluated after 48 h. As colonocytes have greater exposure to the bioavailable IGF-1 in obese condition, in the IGF-1 treatments, the cells were pre-incubated with IGF-1 for 10 minutes followed by the combination treatment to assess the effect of RSV-GSE combination when the cells are already primed to proliferate. Dose response study showed that 25 nM IGF-1 is essential to produce a significant elevation of HCT-116 p53+/+ cell proliferation (data not shown). In case of HT-29 and SW-480 advanced colon cancer cell lines, even 10 nM IGF-1 was sufficient to prime the cells to proliferate (Vanamala et al. 2010). Thus, we used 25 nM concentration of IGF-1 for subsequent experiments in HCT-116 p53 +/+ cells. In the apoptosis experiment, after 24 h of treatment, cells were assayed with the

manufacturer's protocol using the cell death detection ELISA kit using the manufacturer's protocol.

3.3.11 Statistical Analysis

Analysis of variance and Fisher's least square difference at 5 % significance level determined degree of significance between treatments. Pearson correlation at 1 % determined correlation between apoptosis and Bax/Bcl-2 ratio.

3.4 Results

3.4.1 Resveratrol (RSV) or Grape Seed Extract (GSE) Suppressed Cell Proliferation of HCT-116 Cells

Anti-proliferative and cytotoxic effects of RSV or GSE were investigated using HCT-116 p53 +/+ and p53 -/- colon cancer cells. RSV or GSE resulted in suppression of cell proliferation in a dose dependent manner (Figure 3.2A, 3.2B). Our results demonstrated that RSV suppresses the colon cancer cell proliferation in both p53 +/+ and p53 -/- cells (Figure 3.2A), however, cell proliferation suppression was more potent in p53 -/- cells (IC 50 of RSV was 72 μM in p53 -/- cells compared to 110 μM in p53 +/+ cells). Treatment of HCT-116 p53 +/+ cells with 37.5, 50, 75, 100 and 125 μg/ml of GSE resulted in 31-90% cell proliferation suppression (Figure 3.2B). Similar growth inhibition was seen in p53 -/- cells, however the IC 50 for GSE was higher in p53 -/- cells (47 μg/ml compared to 37.5 μg/ml in p53 +/+ cells). Together, the data in Figures 3.2A and 3.2B clearly demonstrate the efficacy of RSV or GSE to inhibit the growth of human colon cancer cells (HCT-116 p53 +/+ and p53 -/-). The cytotoxicity of RSV or GSE towards HCT-116 p53 +/+ and p53 -/- *in vitro* was evaluated using LDH assay. Minimal cytotoxicity was observed

for RSV or GSE even at concentrations up to 100 μM and 100 μg/ml, respectively (Figure 3.2D). Cytotoxicity was compared against the positive control Triton X-100.

3.4.2 RSV or GSE Induced Apoptosis

Induction of apoptosis by RSV or GSE in HCT-116 p53 +/+ and p53 -/- cells was analyzed using nucleosomal fragmentation assay (Cell Death Detection ELISA). RSV induced apoptosis in both p53 +/+ and p53 -/- cells, however, only at higher concentrations close to 100 μM (Figure 3.2C). GSE also induced apoptosis, but mainly in p53 +/+ cells (GSE > 75 μg/ml) (Figure 3.2C). This suggests that low concentrations of RSV and GSE, are unable to induce apoptosis even though they might be able to suppress cell proliferation (Figure 3.2A, 3.2B and 3.2C). Confirmation using a p53 inhibitor (Figure 3.4E), suggest that RSV induced apoptosis in a p53-independent manner; however, GSE requires a functional p53 to induce apoptosis in HCT-116 cells.

3.4.3 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis.

Based on the anti-proliferative and pro-apoptotic properties of RSV or GSE in the HCT-116 cell lines, the effect of the combination of RSV and GSE on the HCT-116 p53 +/+ and p53 -/- cells was analyzed. Possible combinatorial effects (synergy, additive or antagonism) were assessed using a series of nine different combinations (Table 3.1). Data were analyzed using combination index (CI) values by the method of Chou and Talalay (Chou and Talalay 1983). Results indicated that a combination of RSV and GSE resulted in a greater growth inhibition as compared to either agents alone at the same concentrations (Table 3.1, Figure 3.2A and 3.2B) in HCT-116 p53 +/+ cells after 48 h of treatment. Interestingly, seven out of the nine combinations

showed synergism, whereas the other two showed additive effects in p53 +/+ cells (Table 3.1). Elevated cell proliferation suppression was seen in p53 -/- cells, wherein the same seven combinations demonstrated synergism as in p53 +/+ cells (Table 3.1). For the nine combinations, apoptosis was measured, albeit only in p53 +/+ cells. Out of the nine combinations, two combinations (C1: RSV 27 μM and GSE 35 μg/ml and C2: RSV 25 μM and GSE 51 μg/ml) were selected for further experiments based on their ability to adequately suppress cell proliferation, induce apoptosis comparable to RSV doses of 100 μM, low CI values and reduced RSV levels. Low doses of RSV and GSE in combinations C1 and C2 are unable to elicit apoptosis individually in p53 +/+ cells (Figure 3.2C).

3.4.4 RSV-GSE Combination Induced Cell Cycle Arrest in Go/G1 Phase in Both HCT 116 P53 +/+ and P53 -/- Cells

Fluorescence-activated cell sorting analysis (FACS) technique was used to determine the effect of RSV-GSE combination on cell cycle progression in HCT-116 cells. Figures 3.3A and 3.3B illustrate the distribution of HCT-116 p53 +/+ cells and p53 -/- cells in the G_0/G_1, S and G_2/M phases after treatment with the RSV-GSE combination, respectively. RSV-GSE combination had higher percentage of cells in the G_0/G_1 phase compared to control in both p53 +/+ and p53 -/- cells. These results suggest that the RSV-GSE combination targets G_0/G_1 to S phase progression to arrest HCT-116 cell proliferation.

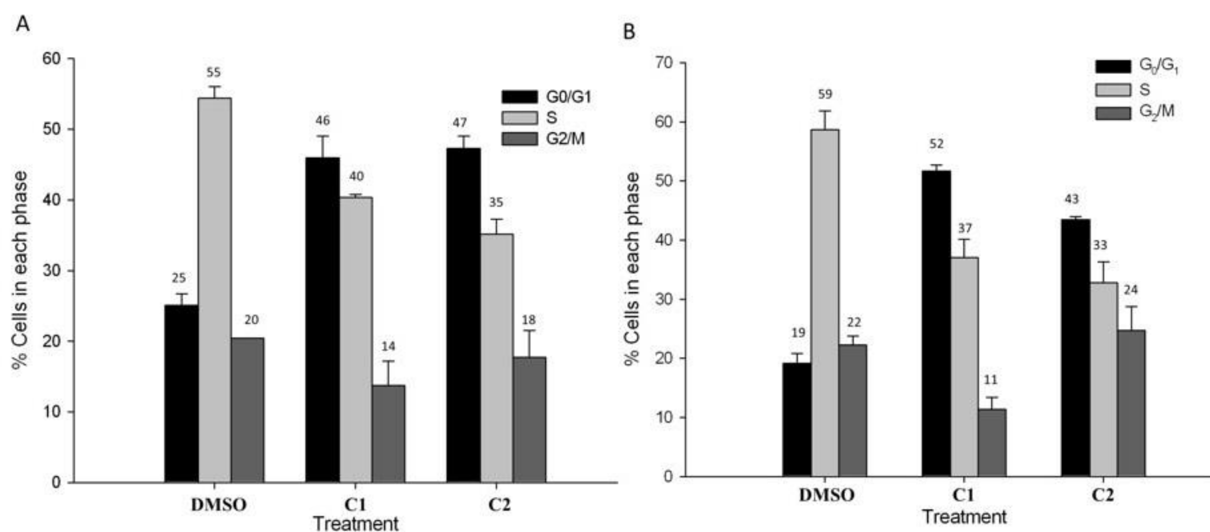

Figure 3.3 Effect of combination of resveratrol (RSV) and grape seed extract (GSE) on cell cycle progression of HCT 116 colon cancer cells. HCT-116 p53 +/+ (A) and p53 -/- (B) cells were plated at a density of 15×10^6 cells per 100 mm plate and after 18 h treatment with the vehicle (DMSO), C1 and C2; they were analyzed by fluorescence-activated cell sorting analysis (FACS). Results were expressed as % of cells in each phase. Numbers on top of the bars represent percent cells in respective phases in each treatment group. C1 and C2 have RSV (27 µM and 25 µM) and GSE (35 and 51 µg/ml), respectively.

3.4.5 RSV-GSE Combination Induced Apoptosis is P53-Dependent

We used nucleosomal fragmentation assay and caspase-glo 3/7 assay to detect whether combination of RSV and GSE induces apoptotic death. There was an increase in apoptosis with the two combination doses (C1 and C2) compared to the control (Figure 3.4A). This is particularly important, since RSV (~ 25 µM) or GSE (~ 37.5 µg/ml) alone do not induce apoptosis at the concentrations present in the combination (Figure 3.2C and Table 3.1). Similar results were obtained when apoptosis was measured using the caspase-glo 3/7 assay confirming the synergy between RSV and GSE (Figure 3.4B).

RSV or GSE alone at high concentrations demonstrated p53 independent and p53 dependent apoptosis respectively, at 24 h time point (Figure 3.2C), however, at the low concentrations used in C1 or C2 neither RSV nor GSE induced apoptosis in p53 +/+ or p53 -/- cells. However, we did see a difference in the induction of apoptosis by RSV-GSE combination in p53 +/+ and p53 -/- cells at 12 h (Figure 3.4A and 3.4B). To confirm, if p53 plays an important role in apoptosis induction by the RSV-GSE combination, cells were treated with RSV and/or GSE for a further 12 h (total 24 h). After 24 h, significant differences were observed between p53+/+ and p53-/- cells in the induction of apoptosis with RSV-GSE combination, suggesting that combination treatment requires a functional p53 to induce apoptosis (Figure 3.4B). Results were confirmed using caspase-glo 3/7 assay (Figure 3.4D). However, increasing GSE concentration to 50 μg/ml did not elevate apoptosis in C2 compared to C1 indicating that once GSE levels reach 50 μg/ml, RSV had a marginal effect to potentiate GSE actions. This also suggests that the effect of synergy is at best when the two compounds are at low concentrations together.

To confirm the role of p53, transcriptional activity of p53 was inhibited using the p53 inhibitor pifithrin-α (40 μM, (Murphy et al. 2004)) and then treated with the combination treatment for 24 h and assayed for apoptosis. HCT-116 p53 +/+ cells treated with the p53 inhibitor acted like the p53 -/- cells, and the RSV-GSE combination did not induce apoptosis in p53 +/+ cells in the presence of the p53 inhibitor (Figure 3.4E). This confirms that the RSV-GSE combination induced apoptosis via p53 related signaling pathways. Further experiments delineating mechanisms of the RSV-GSE combination were only performed in p53 +/+ cells.

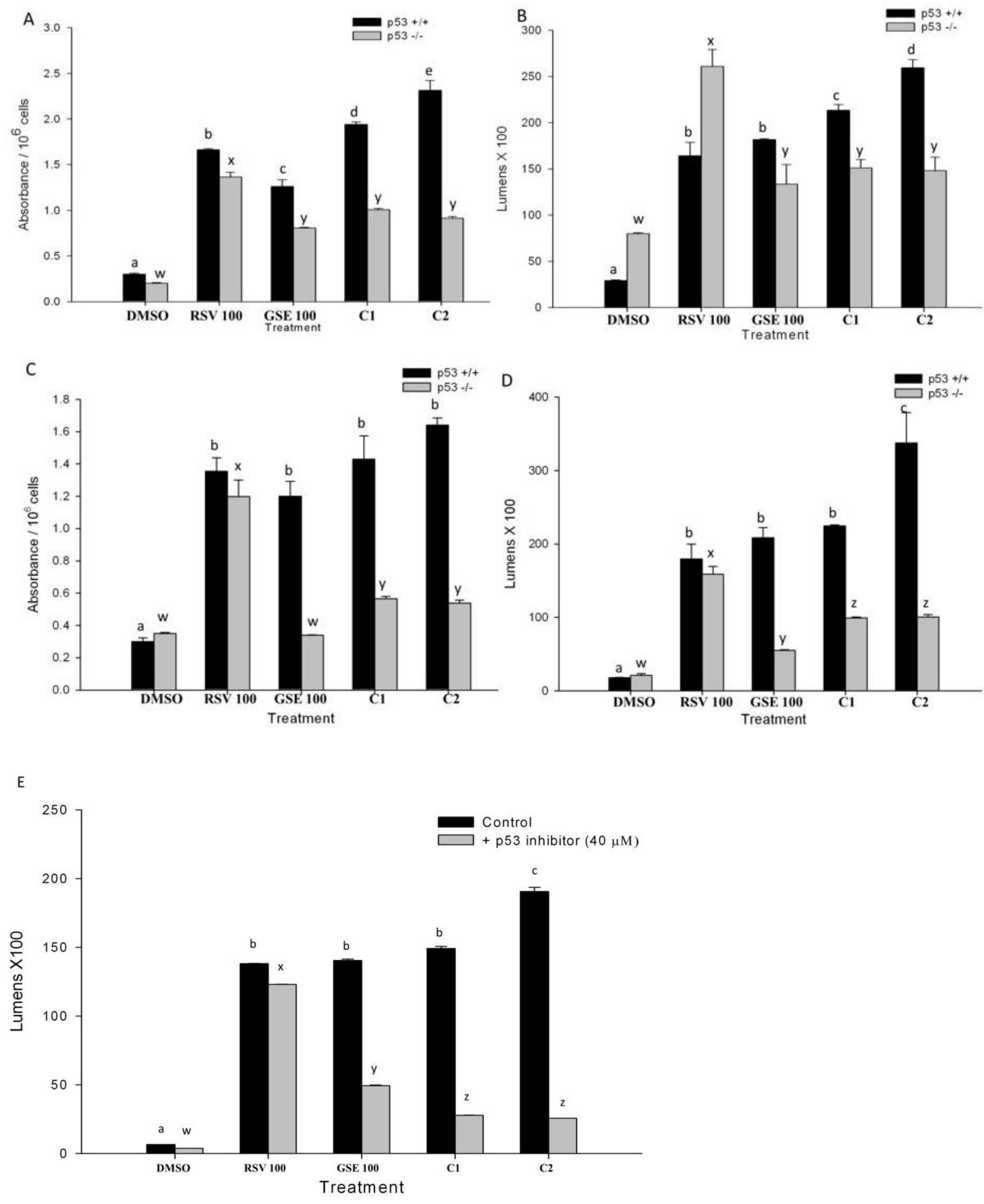

Figure 3.4 Synergistic combination of resveratrol (RSV) and grape seed extract (GSE) induced apoptosis in HCT-116 human colon cancer cell lines. (A, C) HCT-116 p53 +/+ and -/- cells were

treated with RSV and/or GSE for 12 and 24 h and induction of apoptosis was determined by cell death detection ELISA assay. The rate of apoptosis was expressed as absorbance value measured at 405 nm, normalized to 10^6 cells. (B, D) HCT-116 p53 +/+ and -/- cells were treated with RSV and GSE alone and in combination for 12 and 24 h and apoptosis was determined using caspase-glo 3/7 assay. E) HCT-116 p53 +/+ cells were pretreated with the p53 inhibitor pifithrin-α (40 μM) for 45 minutes followed by RSV and/or GSE for 24 h and apoptosis was detected using caspase-glo 3/7 assay. Results were expressed as mean ± SE for three replicate experiments for each treatment group. C1 and C2 have RSV (27 μM and 25 μM) and GSE (35 and 51 μg/ml), respectively. Means that differ by a common letter (a, b, c, d, e for p53 +/+ cells and w, x, y, z for p53 -/- cells or pifithrin-α treatment) differ ($P \leq 0.05$).

3.4.6 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis via Caspase-3 Dependent and Bax/Bcl-2 Pathways.

To investigate whether the induction of apoptosis by RSV-GSE combination in HCT-116 p53 +/+ cells was through activation of p53 downstream pathways, lysates from cells treated with RSV and GSE, alone and in combination, were subjected to western blot analysis. RSV in combination with GSE elevated pp53 (activated p53, ser 15) in HCT-116 p53 +/+ cell lines (Figure 3.5A). This is particularly interesting because RSV or GSE were not as effective in elevating pp53 levels at lower concentrations present in the combination (Figure 3.5A). Levels of Bax and Bcl-2, pro and anti-apoptotic members of the BH3 family of apoptotic proteins were measured in the combination treatment in p53 +/+ cells. Combination of RSV and GSE elevated Bax levels and concomitantly suppressed levels of Bcl-2, overall elevating the Bax:Bcl-2 ratio (Figure 3.5B). Bax:Bcl-2 ratio showed significant correlation with apoptosis in both the cell lines

(Pearson correlation coefficient = 0.878 for p53 +/+ cells, $P \leq 0.01$), indicating the RSV-GSE induced apoptosis might be via the mitochondrial mediated apoptotic pathway (De Angelis et al. 1998).

To further confirm the route of apoptosis, caspase-3 and caspase-8 were inhibited separately and then treated with the combination treatment. We measured apoptosis using the nucleosomal fragmentation assay 24 h post treatment. Treatment with the cell-permeable caspase-3 inhibitor DEVD-CHO (50 μM) suppressed RSV-GSE combination induced apoptosis (Figure 3.5C). In contrast, incubation with the caspase-8 inhibitor IETH-CHO (50 μM) had no significant effect on apoptosis induction by RSV-GSE combination, indicating that the cell death is not through the death receptor-mediated pathway involving caspase-8 activation (Figure 3.5C). 5-florouracil served as the positive control for p53 dependent apoptotic pathway (Sun et al. 2007).

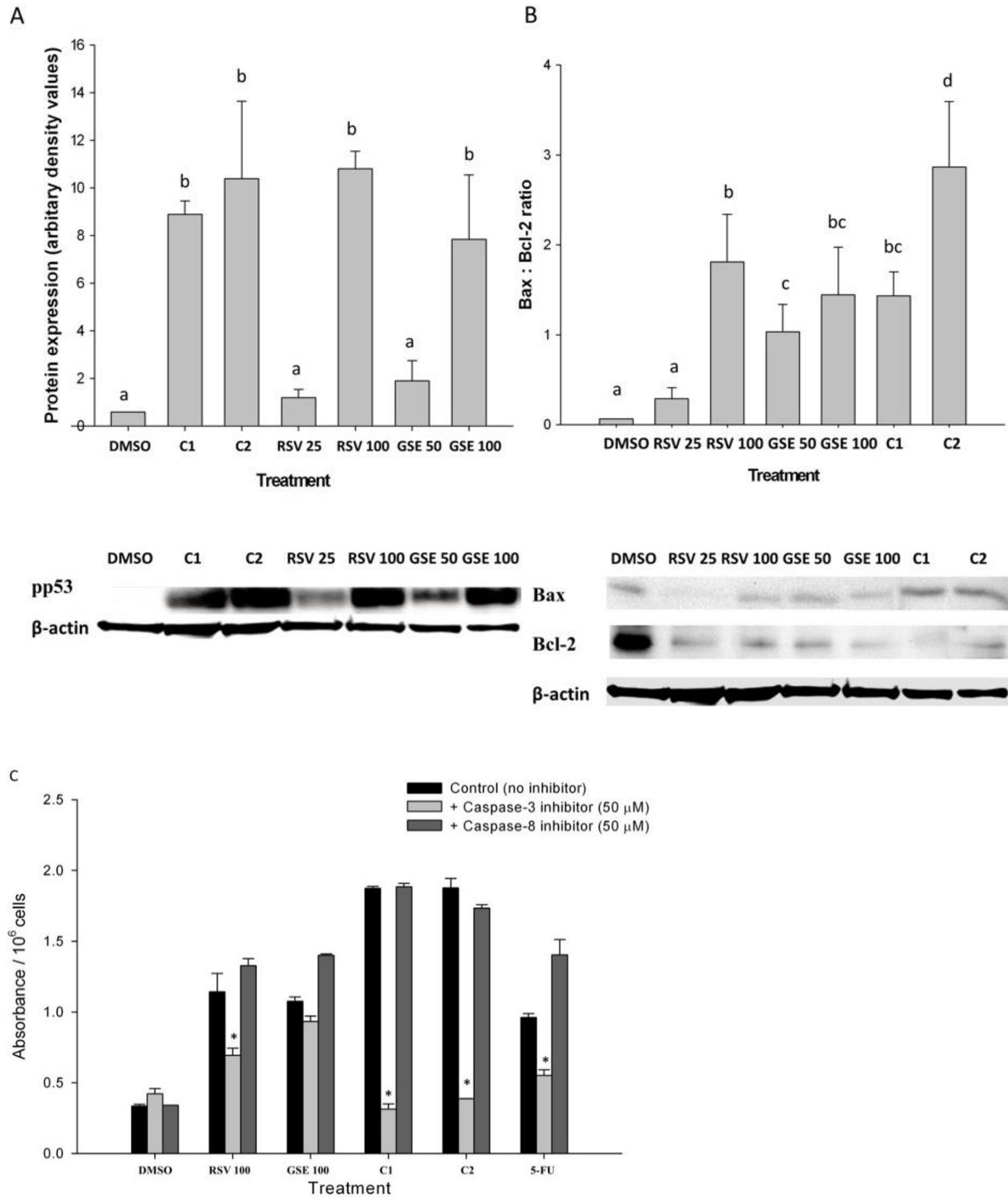

Figure 3.5 Apoptotic induction by the synergistic combination of resveratrol (RSV) and grape seed extract (GSE) involves pathways downstream of p53. HCT-116 p53 +/+ and p53 -/- cells were treated with RSV, GSE and combination of RSV and GSE. C1 and C2 have RSV (27 μM

and 25 μM) and GSE (35 and 51 μg/ml), respectively. Whole cell lysates were analyzed by western blotting for (A) pp53, (B) Bax, Bcl-2 as described in materials and methods. Blots were incubated with the indicated antibodies. Similar results were obtained in duplicate experiments. (C) HCT-116 p53 +/+ cells were treated with cell permeable caspase-3 (50 μM) and caspase-8 (50 μM) inhibitors for 45 minutes followed by RSV and/or GSE and apoptosis was detected using cell death detection ELISA assay. 5-florouracil (5-FU) served as the positive control. RSV 100 and GSE 100 also served as positive control based on earlier reports (Park et al. 2001; Roy et al. 2005). Results were expressed as mean ± SE for three replicate experiments for each treatment group. * indicates significant suppression compared to control (no inhibitor). Means that differ by a common letter (a, b, c, d) differ ($P \leq 0.05$).

3.4.7 N-Acetyl Cysteine (NAC) Inhibited RSV-GSE Combination Induced Apoptosis in HCT-116 P53 +/+ Cells

HCT-116 p53 +/+ cells were pre-incubated with NAC (2 mM), a free radical scavenger and/or catalase (CAT; 100 U/ml), a hydrogen peroxide radical scavenger, for 1 h and then treated with RSV-GSE combination for a total time of 24 h. Both proliferation and apoptosis were measured using WST-1 and caspase-glo 3/7 assays, respectively. In the presence of NAC, RSV-GSE combination induced proliferation arrest, however apoptosis was suppressed. However, CAT could suppress neither proliferation arrest nor apoptosis induced by the combination (Figure 3.6A, 3.6B).

3.4.8 RSV-GSE Combination is Non-Toxic to Normal Cells

To know the effect of RSV-GSE combination on normal cells, CRL-1831 normal colon epithelial cells were treated with the RSV-GSE combination for 24 h and assessed for proliferation and apoptosis using WST-1 and caspase-glo 3/7 assays, respectively. RSV-GSE combination induced neither proliferation arrest nor apoptosis elevation suggesting that the combination is effective against cancer cells with no adverse action on normal cells (Figure 3.6C, 3.6D) *in vitro*.

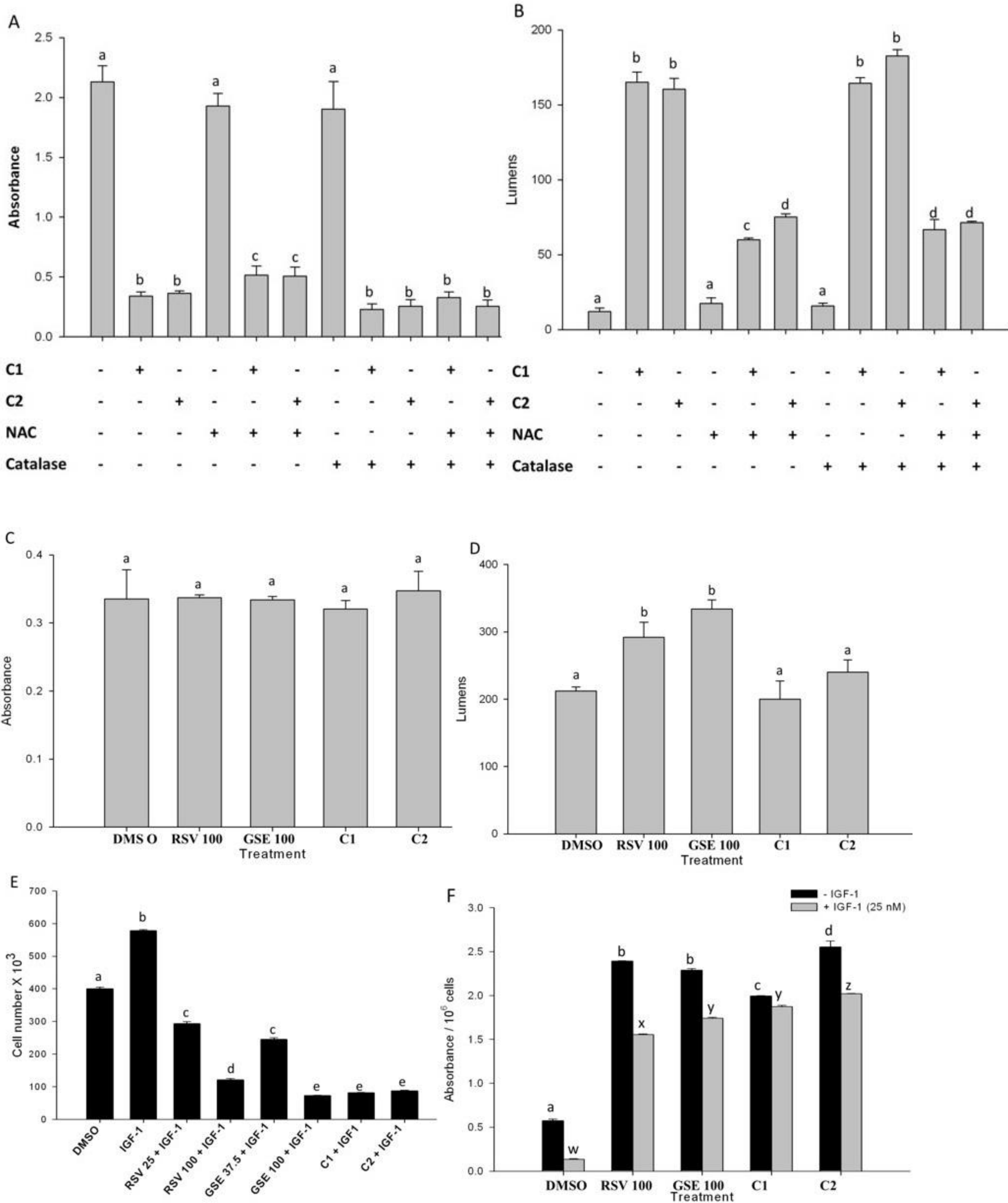

Figure 3.6 N-acetyl cysteine (NAC) suppresses resveratrol (RSV)-grape seed extract (GSE) combination's anti-proliferative (A) and pro-apoptotic (B) properties. HCT 116 p53+/+ and p53 -/- cells were pre-incubated with NAC (2 mM) or catalase (100 U/ml) for 1 hr and then subjected

to the RSV-GSE combination (24 h treatment in total) and assessed for proliferation and apoptosis using WST-1 and caspase-glo 3/7 assay respectively. C-F: RSV-GSE combination is non-toxic to normal cells, but showed anti-proliferative and pro-apoptotic properties even in the presence of insulin like growth factor-1 (IGF-1) in HCT-116 cells. CRL-1831 normal colonic epithelial cells were treated with RSV, GSE alone and in combination and proliferation (48 h) (C) and apoptosis (24 h, D) were measured by WST-1 and caspase-glo 3/7 assay respectively. HCT-116 p53 +/+ cells were primed to proliferate by incubation with IGF-1 (25 nM) for 10 minutes followed by RSV and/or GSE. Cell proliferation (E) was measured using WST-1 assay 48 h after treatment. For apoptosis (F), cells were seeded in 12 well plates pre-treated with IGF-1 (25 nM) followed by treatment with RSV and/or GSE and apoptosis was measured using cell death detection ELISA assay 24 h post treatment. Results were expressed as mean ± SE for three replicate experiments for each treatment group. C1 and C2 have RSV (27 µM and 25 µM) and GSE (35 and 51 µg/ml), respectively. Means that differ by a common letter (a, b, c, d, e, f for p53 +/+ cells and w, x, y, z for IGF-1 treatments) differ ($P \leq 0.05$).

3.4.9 RSV-GSE Combination Suppressed Cell Proliferation and Induced Apoptosis Even in the Presence Of IGF-1

Growth stimulatory effects of IGF-1 and the anti-proliferative effects of RSV-GSE combinations were investigated using HCT-116 p53 +/+ cells. Our results demonstrated that RSV-GSE combinations suppressed colon cancer cell proliferation even when the cells were primed to proliferate with IGF-1. After 48 h, IGF-1 (25 nM) treatment elevated HCT-116 cell proliferation confirming its growth stimulatory effects (Figure 3.6E). Moreover, pre-incubation with IGF-1 (25 nM) for 10 minutes followed by treatment with the combination resulted in

suppressed cell proliferation (Figure 3.6E). Induction of apoptosis by the RSV-GSE combination in the presence of IGF-1 was analyzed using nucleosomal fragmentation assay. The combination treatment induced apoptosis even in the presence of IGF-1 suggesting its role as a potential chemopreventive agent even against IGF-1-promoted colon cancers (Figure 3.6F).

3.5 Discussion

Current strategy of cancer management includes: surgery, radiation therapy and chemotherapy, and in occasional instances investigational drug use on a case by case basis. However, conventional therapy is associated with many side-effects. Hence, methods are designed to improve the efficacy of these treatment modalities or to devise newer ways to treat or even prevent cancer are needed. However, most chemoprevention trials with single active compounds fail to provide satisfactory results. Epidemiological studies showed that plant based diets are associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease (Liu 2004). Phytochemicals possess potent antioxidant and anti-proliferative properties (Liu 2003). We previously showed that RSV at concentrations > 100 μM suppressed colon cancer proliferation even in the presence of IGF-1, a mitogenic growth factor, by inhibiting the critical components in the IGF-1R/Wnt signaling pathway, and elevated apoptosis (Vanamala et al. 2010). However, in fruits and vegetables, bioactive compounds exist as a complex mixture that synergize or complement each other's chemopreventive/protective actions. The results of the present study are in support of such belief since the present study showed that suppression of proliferation and elevation of apoptosis in HCT-116 p53 +/+ human colon cancer cell lines at 100 μM or higher concentrations of RSV could be accomplished at 25 μM, when combined with GSE at doses of 35-50 μg/ml (Table 3.1). These results strongly

support our hypothesis that combining bioactive compounds like RSV and GSE could reduce the dose of either compound while providing similar or better anti-cancer properties *in vitro* lending support to the proposal that combinatorial approach towards colon cancer chemoprevention is a feasible approach. RSV and GSE induced apoptosis in a variety of cancer cell lines (Jang et al. 1997; Agarwal et al. 2002; Banerjee et al. 2002; Bhat and Pezzuto 2002; Laurent et al. 2004; Sharma et al. 2004; Kaur et al. 2006; Sexton et al. 2006; Alkhalaf 2007; Engelbrecht et al. 2007; Kaur et al. 2008; Athar et al. 2009; Vanamala et al. 2010). Even though synergistic combination treatments enhanced apoptosis in both HCT-116 p53 +/+ and p53 -/- cells, at 12 h post treatment, apoptosis was more pronounced in p53 +/+ cells compared to p53 -/- cells, suggesting a significant role for p53. At 24 h post treatment, both the combinations induced apoptosis only in p53 +/+ cells. Involvement of p53 in the enhancement of apoptosis in HCT-116 p53 +/+ by RSV-GSE combination was reaffirmed by the use of p53 transcriptional inhibitor pifithrin-α, that is known to block p53-dependent transcriptional activation and apoptosis.

RSV and GSE demonstrated p53 independent and p53 dependent apoptosis alone at high concentrations at 24 h time point (Figure 3.2C). RSV when used at $\geq$100 μM effectively suppressed proliferation and augmented apoptosis in comparison to the action of GSE when used alone or in combination with RSV in HCT-116 p53 -/- cells. These results indicate that RSV at higher concentrations works via p53 independent pathways to suppress cell proliferation and induce apoptosis. However, in HCT-116 p53 -/- cells, combination of GSE and RSV did enhance apoptosis to a small extent possibly via p53 independent pathways indicating that RSV might potentiate apoptotic action of GSE independent of p53 status of the cells. Overall, the results are still intriguing since the low concentrations of RSV and GSE in C1 and C2 did not induce apoptosis individually in p53 +/+ or p53 -/- cells (Figure 3.2C). It should be noted that C1 and

C2 did not differ significantly in inducing apoptosis indicating that the potentiating effect of RSV is marginal once the GSE concentration reaches 50 µg/ml.

In the present study, RSV-GSE combination induced apoptosis via activating p53 (pp53), elevating Bax and suppressing Bcl-2 (increase in the Bax:Bcl-2 ratio) in p53 +/+ cells, which alters mitochondrial membrane permeability. Such altered mitochondrial membrane permeability releases cytochrome C into the cytosol (Lomonosova and Chinnadurai 2008; Kang and Reynolds 2009) that triggers activation of caspase-9, which accelerates apoptosis by activating other caspases. It is evident from the results of the present study that caspase-3 inhibitor suppressed apoptosis induced by the combination of RSV-GSE while caspase-8 inhibitor had no significant effect on apoptosis, suggesting that the cell death is not through the major death receptor-mediated pathway involving caspase-8 activation.

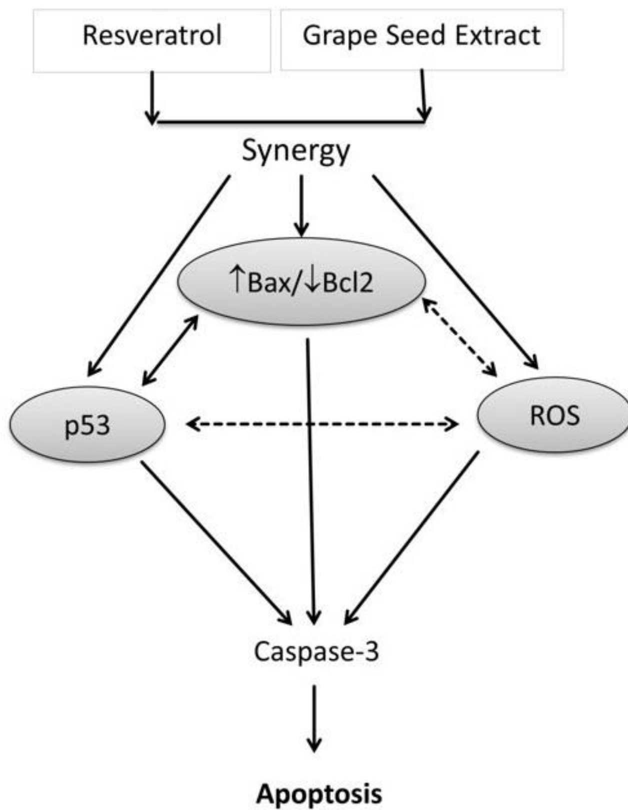

Figure 3.7 Summary of the signaling pathways for resveratrol-grape seed extract (RSV-GSE) combination-induced apoptosis. RSV-GSE combination induced apoptosis involves interplay of reactive oxygen species (ROS), p53, Bax/Bcl-2 and caspase-3.

Several studies have suggested that cancer chemotherapeutic drugs induce apoptosis of tumor cells, in part, by inducing the formation of reactive oxygen species (ROS) (Hou et al. 2005; Low et al. 2010). The p53 tumor suppressor gene can induce either apoptosis or senescence in response to cellular stresses (Shatrov et al. 2000; Macip et al. 2003). ROS accumulation and mitochondrial function can contribute to p53-dependent apoptosis and it has been shown in multiple studies that ROS inducers like bioactive agents collaborate with p53 to influence apoptosis (Tinhofer et al. 2001; Hou et al. 2005; Reddivari et al. 2007; Zhou et al.

2008). Recent articles have pointed out the importance of the Bax, Bcl-2 and ROS production in tumor cells (Chen and Pervaiz 2007). Bcl-2 overexpression, in particular, has shown to suppress apoptosis induced by RSV by a pathway that involves ROS production (Low et al. 2010). Our results indicated that N-acetylcysteine, a free radical scavenger and glutathione precursor, suppressed apoptosis induced by the RSV-GSE combination. However, catalase, an anti-oxidant enzyme that brings about its cytoprotective action by suppressing or neutralizing the toxic hydrogen peroxide radicals, could not inhibit the cytotoxic action of RSV-GSE. These results indicate that this combination (RSV-GSE) does not bring about its actions by augmenting hydrogen peroxide radical formation (Aragon et al. 1992). Since NAC could protect against the combination induced apoptosis, the activation of caspase cascade could be linked to the generation of ROS (Hou et al. 2005). Our results explain that the pathway that the combination induces apoptosis involves an interplay between ROS, p53, Bax/Bcl-2 and caspase-3 (Figure 3.7) (Liu et al. 2008; Low et al. 2010). ROS can act via enhancing mitochondrial membrane permeability and thus can induce apoptosis via the caspase-3 cascade (Hou et al. 2005). Overall, these results are in support that mitochondrial apoptotic pathway is responsible for the observed apoptosis in HCT-116 cells.

Further, RSV-GSE combination did not suppress the proliferation or induce apoptosis of normal colon epithelial cell CRL-1831 line indicating that the RSV-GSE combination preferentially target cancer cells while sparing their normal counterparts. The exact reason for this is not clear but could be due to differential metabolism of bioactive compounds in normal and cancer cells (Lu et al. 2001; Potter et al. 2002; Jayaprakasha et al. 2010) that remains to be established.

The germline adenomatous polyposis coli mutations in colonocytes are highly predictive of colon cancer. Hence, the use of RSV-GSE combination that selectively activates p53 to induce apoptosis in colon cancer but not in normal cells could be a promising dietary approach in its prevention. This is particularly interesting in the light of the known fact that polyps possess intact p53 and colon cancers develop from the polyps. Based on these results, it is important to pursue the chemopreventive and chemoprotective properties of RSV-GSE synergistic combination in both rodent and human models of colon cancer in future studies.

Members of IGFs family and the IGF-binding proteins (IGFBPs) play a critical role in the progression of a variety of cancers during obesity including colon cancer (Dupont et al. 2003). Recently we showed that RSV at ≥ 100 μM concentration could effectively suppress IGF-1 (elevated during obesity) stimulated growth of human colon cancer cell lines via suppression of IGF-1R/PI3K/β-catenin pathway. IGF-1 binding to IGF-1R stimulates downstream proliferating pathways such as the PI3K/Akt (Laurino et al. 2005) and Ras signaling (Desbois-Mouthon et al. 2001) resulting in increased human colon cancer cell proliferation. RSV could effectively suppress IGF-1R levels and downstream PI3K/Akt/beta-catenin signaling. This study, for the first time, demonstrated that RSV-GSE combination suppressed IGF-1 stimulated HCT-116 colon cancer cell proliferation and induced apoptosis suggesting its potential role as a chemopreventive agent against colon cancer even in obese subjects.

In summary, the current study revealed that RSV and GSE act in concert with each other in potentiating their anti-cancer properties in human colon cancer cell line HCT-116 via p53 dependent mechanisms, but not in normal CRL-1831 colon epithelial cells. Thus, these results established the molecular basis for the beneficial effect of RSV-GSE combination that is a popular dietary supplement. It is possible that the combination of RSV-GSE may result in the

formation of a variety of potent bioactive metabolites that possess chemoprotective effect in animal/human models at even lower concentration compared to the present *in vitro* combinations used in the study (Tessitore et al. 2000; Banerjee et al. 2002; Li et al. 2002; Vanamala et al. 2010). However, current knowledge on the effect of RSV-GSE combination is based mainly on our *in vitro* study. In vivo studies are needed to confirm these results, especially in the colon using sporadic/spontaneous colon cancer mice models, so that this system can be manipulated for the prevention and treatment of colon cancer.

Published data

Resveratrol potentiates grape seed extract induced human colon cancer cell apoptosis.

Sridhar Radhakrishnan, Lavanya Reddivari, Robert Sclafani, Undurti N Das, and Jairam Vanamala.

Frontiers in Bioscience E3, 1509-1523, June 1, 2011.

http://www.ncbi.nlm.nih.gov/pubmed/21622155

3.8 References

Agarwal, C., et al. (2002). "Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release." Carcinogenesis **23**(11): 1869-1876.

Agarwal, C., et al. (2007). "Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3'-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells." Carcinogenesis **28**(7): 1478-1484.

Ahmed, R. L., et al. (2007). "Interactions between insulin, body fat, and insulin-like growth factor axis proteins." Cancer Epidemiol Biomarkers Prev **16**(3): 593-597.

Alkhalaf, M. (2007). "Resveratrol-induced apoptosis is associated with activation of p53 and inhibition of protein translation in T47D human breast cancer cells." Pharmacol **80**(2-3): 134-143.

American Cancer Society, I. (2009, 05/18/2009). "Detailed Guide: Colon and Rectum Cancer. What Are the Key Statistics for Colorectal Cancer?", from http://www.cancer.org/docroot/cri/content/cri_2_4_1x_what_are_the_key_statistics_for_colon_and_rectum_cancer.asp.

Aragon, C. M., et al. (1992). "Ethanol metabolism in rat brain homogenates by a catalase-H2O2 system." Biochem Pharmacol **44**(1): 93-98.

Asensi, M., et al. (2002). "Inhibition of cancer growth by resveratrol is related to its low bioavailability." Free Radic Biol Med **33**(3): 387-398.

Athar, M., et al. (2009). "Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms." Arch Biochem Biophys **486**(2): 95-102.

Aziz, M. H., et al. (2003). "Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review)." Int J Oncol **23**(1): 17-28.

Banerjee, S., et al. (2002). "Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9." Cancer Res **62**(17): 4945-4954.

Bhat, K. P. and J. M. Pezzuto (2002). "Cancer chemopreventive activity of resveratrol." Ann N Y Acad Sci **957**: 210-229.

Chen, Z. X. and S. Pervaiz (2007). "Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells." Cell Death Differ **14**(9): 1617-1627.

Chou, T. and P. Talalay (1983). "Analysis of combined drug effects: a new look at a very old problem." Trends Pharmacol Sci **4**: 450-454.

Davies, M., et al. (2006). "The insulin-like growth factor system and colorectal cancer: clinical and experimental evidence." Int J Colorectal Dis **21**(3): 201-208.

De Angelis, P. M., et al. (1998). "Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal carcinomas, and association with p53 genotype/phenotype." Mol Pathol **51**(5): 254-261.

Desbois-Mouthon, C., et al. (2001). "Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation." Oncogene **20**(2): 252-259.

Dupont, J., et al. (2003). "The insulin-like growth factor axis in cell cycle progression." Horm Metab Res **35**(11-12): 740-750.

Engelbrecht, A. M., et al. (2007). "Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line." Cancer Lett **258**(1): 144-153.

Frystyk, J. (2004). "Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis." Growth Horm IGF Res **14**(5): 337-375.

Galluzzi, L., et al. (2008). "Targeting p53 to mitochondria for cancer therapy." Cell Cycle **7**(13): 1949-1955.

Grady, W. M. and J. M. Carethers (2008). "Genomic and epigenetic instability in colorectal cancer pathogenesis." Gastroenterol **135**(4): 1079-1099.

Hope, C., et al. (2008). "Low concentrations of resveratrol inhibit Wnt signal throughput in colon-derived cells: implications for colon cancer prevention." Mol Nutr Food Res **52 Suppl 1**: S52-61.

Hou, D. X., et al. (2005). "Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway." Arch Biochem Biophys **440**(1): 101-109.

Jang, M., et al. (1997). "Cancer chemopreventive activity of resveratrol, a natural product derived from grapes." Science **275**(5297): 218-220.

Jang, M. and J. M. Pezzuto (1999). "Cancer chemopreventive activity of resveratrol." Drugs Exp Clin Res **25**(2-3): 65-77.

Jayaprakasha, G. K., et al. (2010). "Bioactive compounds from sour orange inhibit colon cancer cell proliferation and induce cell cycle arrest." J Agric Food Chem **58**(1): 180-186.

Kang, M. H. and C. P. Reynolds (2009). "Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy." Clin Cancer Res **15**(4): 1126-1132.

Kaur, M., et al. (2006). "Grape seed extract induces anoikis and caspase-mediated apoptosis in human prostate carcinoma LNCaP cells: possible role of ataxia telangiectasia mutated-p53 activation." Mol Cancer Ther **5**(5): 1265-1274.

Kaur, M., et al. (2008). "Grape seed extract induces cell cycle arrest and apoptosis in human colon carcinoma cells." Nutr Cancer **60 Suppl 1**: 2-11.

Khanna, S., et al. (2001). "Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract." Free Radic Biol Med **31**(1): 38-42.

Laurent, C., et al. (2004). "Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells." J Agric Food Chem **52**(11): 3301-3308.

Laurino, L., et al. (2005). "PI3K activation by IGF-1 is essential for the regulation of membrane expansion at the nerve growth cone." J Cell Sci **118**(Pt 16): 3653-3662.

LeRoith, D. and C. T. Roberts, Jr. (2003). "The insulin-like growth factor system and cancer." Cancer Lett **195**(2): 127-137.

Li, Z. G., et al. (2002). "Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol." Carcinogenesis **23**(9): 1531-1536.

Lin, J., et al. (2009). "Vitamins C and E and beta carotene supplementation and cancer risk: a randomized controlled trial." J Natl Cancer Inst **101**(1): 14-23.

Liu, B., et al. (2008). "ROS and p53: a versatile partnership." Free Radic Biol Med **44**(8): 1529-1535.

Liu, R. H. (2003). "Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals." Am J Clin Nutr **78**(3 Suppl): 517S-520S.

Liu, R. H. (2004). "Potential synergy of phytochemicals in cancer prevention: mechanism of action." J Nutr **134**(12 Suppl): 3479S-3485S.

Lomonosova, E. and G. Chinnadurai (2008). "BH3-only proteins in apoptosis and beyond: an overview." Oncogene **27 Suppl 1**: S2-19.

Low, I. C., et al. (2010). "Bcl-2 modulates resveratrol-induced ROS production by regulating mitochondrial respiration in tumor cells." Antioxid Redox Signal **13**(6): 807-819.

Lu, J., et al. (2001). "Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts." Carcinogenesis **22**(2): 321-328.

Ma, J., et al. (1999). "Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3." J Natl Cancer Inst **91**(7): 620-625.

Macip, S., et al. (2003). "Influence of induced reactive oxygen species in p53-mediated cell fate decisions." Mol Cell Biol **23**(23): 8576-8585.

Murphy, P. J., et al. (2004). "Pifithrin-alpha inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation." J Biol Chem **279**(29): 30195-30201.

Nassiri-Asl, M. and H. Hosseinzadeh (2009). "Review of the pharmacological effects of Vitis vinifera (Grape) and its bioactive compounds." Phytother Res **23**(9): 1197-1204.

Park, J. W., et al. (2001). "Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity." Carcinogenesis **22**(10): 1633-1639.

Potter, G. A., et al. (2002). "The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1." Br J Cancer **86**(5): 774-778.

Reddivari, L., et al. (2007). "Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways." Carcinogenesis **28**(10): 2227-2235.

Roy, A. M., et al. (2005). "Grape seed proanthocyanidins induce apoptosis through p53, Bax, and caspase 3 pathways." Neoplasia **7**(1): 24-36.

Sexton, E., et al. (2006). "Resveratrol interferes with AKT activity and triggers apoptosis in human uterine cancer cells." Mol Cancer **5**: 45.

Sharma, G., et al. (2004). "Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells." Breast Cancer Res Treat **85**(1): 1-12.

Shatrov, V. A., et al. (2000). "Adenovirus-mediated wild-type-p53-gene expression sensitizes TNF-resistant tumor cells to TNF-induced cytotoxicity by altering the cellular redox state." Int J Cancer **85**(1): 93-97.

Sun, X. X., et al. (2007). "5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction." J Biol Chem **282**(11): 8052-8059.

Tessitore, L., et al. (2000). "Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression." Carcinogenesis **21**(8): 1619-1622.

Tinhofer, I., et al. (2001). "Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2." FASEB J **15**(9): 1613-1615.

Vanamala, J., et al. (2010). "Resveratrol suppresses IGF-1 induced human colon cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways." BMC Cancer **10**: 238.

Vanamala, J., et al. (2008). "Obesity-enhanced colon cancer: functional food compounds and their mechanisms of action." Curr Cancer Drug Targets **8**(7): 611-633.

Weber, H. A., et al. (2007). "Comparison of proanthocyanidins in commercial antioxidants: grape seed and pine bark extracts." J Agric Food Chem **55**(1): 148-156.

Wei, E. K., et al. (2005). "A prospective study of C-peptide, insulin-like growth factor-I, insulin-like growth factor binding protein-1, and the risk of colorectal cancer in women." Cancer Epidemiol Biomarkers Prev **14**(4): 850-855.

Yang, J. and R. H. Liu (2009). "Synergistic effect of apple extracts and quercetin 3-beta-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro." J Agric Food Chem **57**(18): 8581-8586.

Zhou, J., et al. (2008). "Andrographolide sensitizes cancer cells to TRAIL-induced apoptosis via p53-mediated death receptor 4 up-regulation." Mol Cancer Ther **7**(7): 2170-2180.

CHAPTER IV

PURPLE-FLESHED POTATOES REVERSED HIGH-FAT DIET ELEVATED OXIDATIVE STRESS AND INNATE INFLAMMATION MARKERS IN A PIG MODEL.

4.1 Summary

Intake of anthocyanins and anthocyanin-rich fruits has shown to suppress risk of colon inflammation, colon cancer and other chronic diseases. Although consumption of white-fleshed potatoes declined, consumption of color-fleshed potatoes, rich in anthocyanins and other bioactive compounds, increased by 17 % in the US in 2009-10. This may be partly attributed to the putative health benefits of anthocyanins and other bioactive constituents. However, little information exists if purple-fleshed potatoes, rich in anti-oxidant and anti-inflammatory anthocyanins, even after processing, retain their health benefiting properties *in vivo*. We hypothesized that processed (chipped) purple-fleshed potatoes will retain bioactivity and suppress high-fat diet (HFD) elevated oxidative stress/inflammation biomarkers in a pig model. We conducted a study, where 40 pigs (12 weeks on HFD, n=8/group) consumed HFD or HFD containing 10 or 20 % w/w purple or white-fleshed potato chips for an additional 5 weeks. Analytical (UPLC-MS) data on purple and white-fleshed potato chips showed significant differences in metabolite profiles between the two cultivars. In the animal study, the purple-fleshed potato diets had no effect on food intake and weight gain at the end of the study. In the distal colon, only the purple-fleshed potato consuming animals (P10) had elevated GSH:GSSG ratio (oxidative stress marker, P = 0.05) and lower expression of inflammatory markers (P10 and P20; NF-κB, P = 0.05; TNF-α, P = 0.05; TLR-2, P = 0.02; and TLR-4, P = 0.02) compared to

HFD controls; levels in the white-fleshed potato consuming animals were not significantly different from the HFD control animals. Similar results were obtained in the mesenteric fat, as only the purple-fleshed potato consuming animals (P10) had greater GSH:GSSG ratio and adiponectin expression ($P = 0.1$) and lower TNF-α expression (P10 and P20, $P = 0.05$) compared to the HFD control animals. Systemic markers of oxidative stress were suppressed in the purple-fleshed group P10 compared to control. Results of this intervention study suggest that the consumption of purple-fleshed potato chips reversed HFD elevated oxidative and inflammatory biomarkers in the distal colon, mesenteric fat and the systemic circulation; lending support to the health benefits of purple-fleshed potato consumption.

4.2 Introduction

Consumption of a diet rich in fat and low in anti-oxidants is linked to chronic low-grade oxidative stress and inflammation that are conducive for development of disorders like colon cancer (Erdelyi, Levenkova et al. 2009). Animals provided with high-fat diet (HFD) have demonstrated elevated oxidative stress in various tissues and in systemic circulation (Wisse 2004, Berg and Scherer 2005, Shoelson, Herrero et al. 2007, Erdelyi, Levenkova et al. 2009). Indeed, reactive oxygen species (ROS) production was upregulated in the adipose tissue (and the liver) of C57BL/6J mice consuming a HFD (Matsuzawa-Nagata, Takamura et al. 2008). In a Western diet consuming mouse model of sporadic colon cancer, oxidative stress and altered immune responses occurred long before tumors in the colon (Erdelyi, Levenkova et al. 2009).

Oxidative stress and inflammatory processes are very well connected as elevated oxidative stress can activate inflammatory pathways via the NF-κB signaling (Solinas and Karin 2010). Prolonged or chronic, low grade inflammation in the gut can create a local tissue

microenvironment where ROS are released from inflammatory cells. ROS can cause DNA damage and elevate risk for colorectal cancer (Pot, Geelen et al. 2010). Studies have shown that HFD consuming rodent models have dysregulated gut microbiota with greater numbers of harmful bacteria (Kim, Gu et al. 2012) and elevated gut inflammation (Ding, Chi et al. 2010, Kim, Gu et al. 2012). HFD induced altered gut bacteria and TLR activation are key elements for induction of the innate inflammatory response via triggering signaling cascades including the transcription factor NF-κB (Takeda and Akira 2004). Indeed, mice consuming HFD had elevated colonic inflammation indexed by the elevated expression of pro-inflammatory cytokines, the induction of TLR-4, iNOS, COX-2, and the activation of NF-κB compared to the control mice (Kim, Gu et al. 2012).

Current body of evidence supports the role of the gut in visceral fat dysfunction (Drouet, Dubuquoy et al. 2012). Colonic inflammation and altered gut bacterial diversity results in an increase in paracellular permeability of the colon and subsequent translocation of harmful bacteria and bacterial products (LPS, flagellin etc.) into the adjacent mesenteric fat (Gambero, Marostica et al. 2007, Lam, Mitchell et al. 2011). Indeed, in animal and human models of Crohns disease, an inflammatory condition of the colon, the mesenteric fat gets inflamed. Inflamed mesenteric fat exhibited elevated pro-inflammatory gene expression and cytokine production, adipocyte hypertrophy, leading to macrophage infiltration and activation over time (Gambero, Marostica et al. 2007, Maury and Brichard 2010, Lam, Mitchell et al. 2011). The adipose-derived cytokines and increased fatty acid flux from the expanded fat mass have been associated to systemic dysfunction and feed forward to further exacerbate the intestinal and adipose dysfunction – creating a vicious cycle (Lam, Mitchell et al. 2011, Drouet, Dubuquoy et al. 2012).

Elevated adipose inflammation can contribute to elevated circulating inflammatory markers like TNF-α and IL-6 (Arkan, Hevener et al. 2005, Bastard, Maachi et al. 2006, Shoelson, Herrero et al. 2007). TNF-α is over expressed in adipose tissue and plays an important role in mediating the insulin resistance (Berg and Scherer 2005). In addition, HFD consuming animal models had elevated ROS production in the adipose tissue (Matsuzawa-Nagata, Takamura et al. 2008), which can damage lipids. In particular, unsaturated fatty acids are susceptible to ROS damage leading to formation of reactive intermediates such as 8-isoprostane and malondialdehyde, biomarkers in urine/serum for measuring systemic oxidative stress (Apel and Hirt 2004).

Foods abundant in bioactive polyphenols have demonstrated anti-oxidant and anti-inflammatory properties in multiple models both *in vitro* and *in vivo* (Rossi, Serraino et al. 2003, Pan, Lai et al. 2009, Chuang and McIntosh 2011). Several studies have demonstrated an inhibitory effect of polyphenols on the NF-κB activation pathways in intestinal models of inflammation (Hogan, Canning et al. 2010, Umesalma and Sudhandiran 2010, Chuang and McIntosh 2011, Piberger, Oehme et al. 2011). Most studies, however, have been conducted with individual standard polyphenols or plant extracts. However epidemiological studies supporting an anti-inflammatory role for polyphenols are on whole foods including fruits and vegetables, that contain complex mixtures of polyphenols (Romier-Crouzet, Van De Walle et al. 2009).

The potato (*Solanum tuberosum* L.) is the world's 4th largest food crop with over 300 million tons of global production per year. The potato is the most consumed vegetable crop in the US with per capita consumption of approximately 52 kg/person/year (NPC 2010). Due to its high consumption, it is considered as the third largest source of phenolic compounds in the human diet after oranges and apples (Chun, Kim et al. 2005). Purple-fleshed potatoes are rich in

phenolic acids and anthocyanins. We and others have shown that phenolic acids are present at amounts 5-12 times higher in purple-fleshed potatoes compared to white-fleshed potatoes (Rodriguez-Saona, Giusti et al. 1998, Brown 2005, Madiwale, Reddivari et al. 2011, Radhakrishnan, Reddivari et al. 2011). Recently, we reported that purple-fleshed potatoes were more efficient than white- or yellow-fleshed potatoes in suppressing proliferation and elevating apoptosis in early (HCT-116) and advanced (HT-29) stage human colon cancer cell lines (Madiwale, Reddivari et al. 2011, Madiwale, Reddivari et al. 2012). However, unlike other anthocyanin-rich sources such as blueberries, potatoes are almost always processed before consumption (Brown 2005, Walton, Lentle et al. 2006, NPC 2007). Thus, it is important to test if processed purple-fleshed potatoes exert anti-oxidant and anti-inflammatory properties *in vivo*, as this aspect has received little consideration.

To determine extent to which purple-fleshed potatoes, even after chipping, suppress HFD elevated oxidative stress and inflammatory markers *in vivo* compared to white-fleshed potato, 40 pigs (on HFD for 12 weeks; > 130 kg) were provided with HFD and HFD containing 10 or 20 % purple or white-fleshed potato chips for additional 5 weeks (n = 8). We hypothesized that dietary supplementation with 10-20 % purple-fleshed potato chips will reverse the elevated oxidative stress/inflammation biomarkers in the distal colon, mesenteric fat and systemic circulation compared to the HFD consuming controls. We used the pig as an animal model due to similar nutrient metabolism, and gut anatomy, and physiology to humans (Cooper, Berry et al. 1997, Pond and Lei 2000). Evaluation of the anti-oxidant and anti-inflammatory effects of purple-fleshed potato bioactive compounds in HFD consuming pigs might provide evidence for the health benefits of potato consumption and increase market for specialty potato cultivars.

4.3 Materials and Methods

All protocols for the use of animals in this study were approved by both the Colorado State University Institutional Animal Care and Use Committee (CSU-IACUC) and the North Carolina State University Animal Care and Use Committee. CSU-IACUC Protocol approval number 08-318A-03.

4.3.1 Animals

Forty male pigs, (3 weeks post weaning, breed: SPG), were obtained from Murphy-Brown LLC (Warsaw, NC) and were individually housed in solid concrete floor indoor pens at the North Carolina State University Swine Educational Unit (Raleigh, NC). Pigs were provided at 6 weeks age with HFD (C, Table 4.1) for 12 weeks until they are ~130 kg. The animals were grouped, so that mean initial body weight was similar among the treatment groups ($n = 8$ animals/treatment). Initial average body weight was 133.3 ± 1.2 kg. All the animals completed the study and no adverse side effects were reported.

4.3.2 Experimental Diets

After 12 weeks of consuming HFD, pigs were assigned to one of the 5 iso-caloric high-fat dietary groups; Control (no potato), and purple- and white-fleshed potato chips at 10 and 20 % of the diet (w/w). Purple-fleshed potatoes (Purple Majesty) and white-fleshed (Atlantic) potatoes were grown at Black Gold Farms (Pearsall, TX), and were chipped and fried in the industrial collaborators facility at H. E. B. (San Antonio, TX). We have previously characterized the anthocyanin, phenolic acid and anti-oxidant properties of Purple Majesty and Atlantic potatoes used in this study (Madiwale 2012). The potato chips were analyzed using proximate

analysis (IEH-Warren Laboratory, Greeley, CO) before incorporation into diet to ensure that macronutrient composition was similar across the groups. Composition of all the diets is presented in Table 4.1. Pigs consumed the experimental diets for 5 weeks (35 days); the feed and drinking water were provided ad libitum. Body weight, weight gain and feed intake were measured every week during the experimental feeding period.

Table 4.1 Composition of diets used in the study.

	Control	Purple Chips		White Chips	
Ingredients (%)	**C**	**P10**	**P20**	**W10**	**W20**
White Corn	72.80	65.80	58.80	65.80	58.80
Purple-fleshed Potato Chips	0.00	10.00	20.00	0.00	0.00
White-fleshed Potato Chips	0.00	0.00	0.00	10.00	20.00
Soybean meal w/o Hulls	15.00	15.00	15.00	15.00	15.00
Trace Mineral Premix	0.15	0.15	0.15	0.15	0.15
Salt	0.32	0.32	0.32	0.32	0.32
NCSU Vit AgPro	0.03	0.03	0.03	0.03	0.03
Poultry Fat	5.00	3.50	2.00	3.50	2.00
Dry Fat	5.00	3.50	2.00	3.50	2.00
Di-Calcium Phosphate	1.00	1.00	1.00	1.00	1.00
Limestone	0.70	0.70	0.70	0.70	0.70
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Calculated Composition (% unless mentioned)					
Dry Matter	90.5	91.0	91.6	91.0	91.6
Metabolizable Energy, Cal/kg	3810	3800	3800	3800	3800
Crude Protein	13.2	13.0	12.8	13.0	12.8
Lysine	0.64	0.65	0.66	0.65	0.66

Methionine	0.47	0.47	0.46	0.47	0.46
Tryptophan	0.14	0.14	0.14	0.14	0.14
Threonine	0.49	0.48	0.48	0.48	0.48
Crude Fat	12.91	12.93	12.95	12.93	12.95
Calcium	0.60	0.60	0.60	0.60	0.60
Available Phosphorus	0.23	0.23	0.23	0.23	0.23
Phosphorous	0.49	0.48	0.48	0.48	0.48

C: High-fat diet control, P10 and P20 - purple-fleshed potato chips at 10 and 20 % of diet w/w and W10 and W20 - white-fleshed potato chips at 10 and 20 % of the diet w/w.

4.3.3 Blood, Urine and Tissue Collection

The animals were euthanized at the end of study using a captive bolt method. At termination, jugular vein blood was collected without anticoagulant and serum was stored at −80 °C for biochemical analysis. Urine was collected at the end of the study in falcon tubes. Once collected, urine samples were stored at −20 °C for a day and transferred to −80 °C for long term storage until analysis. The distal colon was resected and cleaned with RNAse-free phosphate-buffered saline. The mucosa was scraped using a glass slide into an RNAse free tube, immediately snap frozen in liquid nitrogen, and later transferred to −80 °C for long term storage. Mesenteric fat was collected, cleaned with RNAse free phosphate buffered saline, snap frozen in liquid nitrogen and stored at −80 °C prior to analysis.

4.3.4 Tissue Oxidative Stress

Glutathione levels (GSH: reduced and GSSG: oxidized) were measured in the distal colon and the mesenteric fat using UPLC/PDA (Waters ACQUITY UPLC H-class system) according to the HPLC method of Yilmaz et al (Yilmaz, Keser et al. 2009) with minor modifications. In particular, the protocol (solvent flow rate, total run time) was modified for the UPLC system using the Waters Empower 3 software.

4.3.5 Real Time Polymerase Chain Reaction

Real-time PCR analysis was used to measure the gene expression of pro-inflammatory and anti-inflammatory markers in the distal colon mucosa and the mesenteric fat. Total RNA was extracted using the Phenol-Free Total RNA Purification Kit (Amresco, Solon, OH) and cDNA synthesis was carried out using the qScript™ cDNA SuperMix (Quanta Biosciences,

Gaithersburg, MD), according to the manufacturer's instructions. Real-time PCR was performed using qPCR SYBR Green Supermix (Quanta Biosciences) on an Illumina Eco instrument (San Diego, CA). Messenger RNA expression of genes was calculated using the Eco and Eco Study software (Illumina). Primers used for the study are presented in Table 4.2.

Table 4.2 Primers used in the manuscript for real time PCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Product Length (bp)
Actin	GGCCGGGACCTGACCGACTA	GGAGGAGGAGGAGGCGGCC	162
Adiponectin	GCGAATGGGCATGTTAGGGA	AAATCCGGGGCAGAAAAGGA	107
COX-2	CATTGATGCCATGGAGCTGTA	CTCCCCAAAGATGGCATCTG	70
IL-10	TGATGGGGAGGATATCAAGG	TGATGGGGAGGATATCAAGG	150
NF-κB	CCCATGTAGACAGCACCACCTATGAT	ACAGAGGCTCAAAGTTCTCCACCA	132
TGF-β	CGAGCCCTGGATACCAACTA	AGGCTCCAGATGTAGGGACA	164
TLR-2	CAGTCCGGAGGTTGCATATT	ATGCTGTGAAAGGGAACAGG	137
TLR-4	ATGGCCTTTCTCTCCTGCCTGA	AGGTCCAGTATCTTGACTGATGTGGG	139
TNF-α	ATGGATGGGTGGATGAGAAA	TGGAAACTGTTGGGGAGAAG	151

4.3.6 Systemic Oxidative Stress Markers

Serum samples were analyzed for oxidative stress markers, 8-isoprostane – free and bound, and malondialdehyde using an ELISA and Thiobarbituric Acid Reactive Substances (TBARS) assay kit, respectively, following the manufacturer's protocol (Cayman, Ann Harbor, MI). The oxygen radical absorbance capacity (ORAC) value of serum was calculated based on the previously published method (Huang, Ou et al. 2002). Urine samples were analyzed for oxidative stress markers 8-isoprostane and DNA adduct 8-hydroxy-2′-deoxyguanosine (8-OHDG) following manufacturer's protocol (Cayman). Urinary concentrations were normalized to creatinine levels measured using a colorimetric kit (Cayman).

4.3.7 Serum Inflammatory Markers

Inflammatory markers, TNF-α (Swine ELISA, Life Technologies, Grand Island, NY), leukotriene B4 (LTB4, Cayman), lipopolysaccharide (LPS, Limulus Amebocyte Lysate Kit, Lonza, Basel Switzerland) and acute phase C-reactive protein (CRP ELISA; Life Diagnostics, West Chester, PA) were analyzed in the serum based on the manufacturer's protocols.

4.3.8 Statistical Analysis

Completely randomized block design was used in this study. Individual pigs were the experimental unit. Data were analyzed using the MIXED procedure in SAS (SAS Institute, Cary, NC). $P \leq 0.05$ was considered significant. The results were expressed as means ± S.E. for each treatment group.

4.4 Results

4.4.1 UPLC-MS Profile of Phenolic Compounds in Potato Chips

Typical total ion chromatograms (TIC, Figure 4.1A) illustrated differences between the purple (top panel) and the white-fleshed potato chip extracts. From the LC-MS analysis, a total of 2352 ions characterized by a mass to charge ratio and a retention time were represented as points on the volcano plot (Figure 4.1B). Each ion on this graph is represented according to its P-value (y-axis) and fold change (x-axis). Features/ions located on the right indicate those which are abundant in purple-fleshed potato; and those on the left indicate that are abundant in the white-fleshed potato. We gated the graph (in red lines) using a P value ≤ 0.01 to be significantly altered and fold change of 4 between two varieties. Thus, 466 features were significantly higher in purple-fleshed potato compared to white-fleshed potato, whereas only 62 features were higher in the white-fleshed potato (P value ≤ 0.01, fold change > 4). 825 features were not significantly different between the two potato varieties whereas 999 features were significantly different ($P \leq 0.01$) but fold change difference between varieties was ≤ 4. Thus, we observed that number of significant features with higher abundance was more in the purple-fleshed potato compared to the white-fleshed potato. Peak annotations using METLIN metabolite database were presented in Table 4.3. Phenolic acids (chlorogenic acid and p-coumaric acid) were detected in both white and purple-fleshed varieties; however, the relative abundance was higher in the purple-fleshed potato. Glycosylated anthocyanins were only detected in the purple-fleshed potato and are presented in Table 4.3.

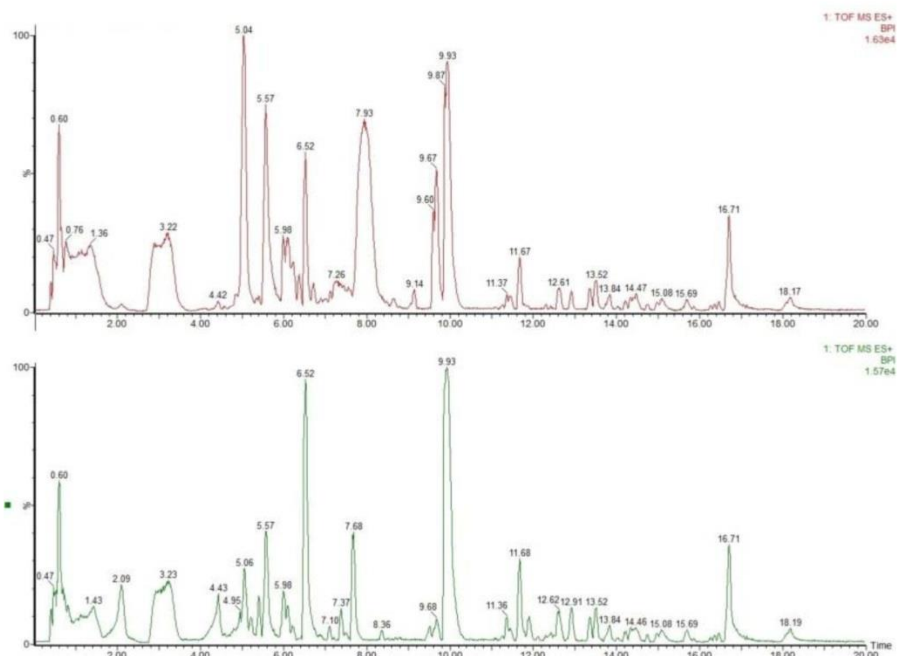

Figure 4.1A Typical total ion chromatograms from LC/MS analysis of purple- (top) and white- (bottom) fleshed potato phenolic extracts.

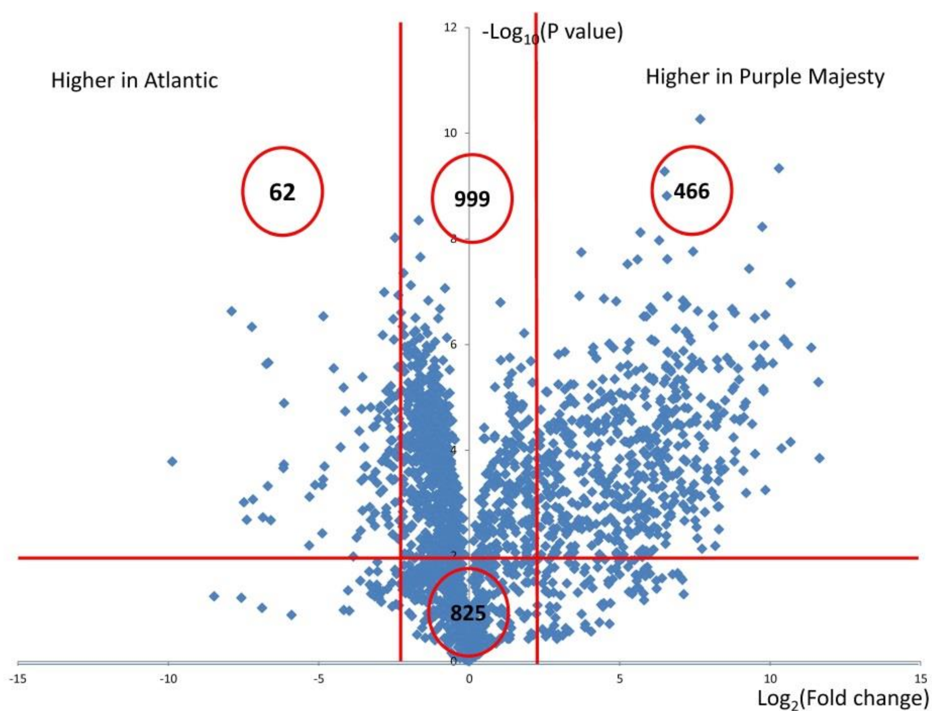

Figure 4.1B Volcano plot of the 2352 features (mass by charge ratio/retention time) obtained after comparison of the UPLC/MS metabolite profile between purple-(Purple Majesty) and

white-(Atlantic) fleshed potato varieties. Each point (ion) on this graph is represented according to its $-\text{Log}_{10}$ (P value) on the y-axis and Log_2 (fold change) on the x-axis. The plot was gated (red lines) using a P value ≤ 0.01 and a fold change ≥ 4 to display differences between the two varieties. Numbers in red circles indicate number of features in each gated areas.

Table 4.3 Phenolic and anthocyanin composition of potato chips by UPLC/MS

Compound Identity	Molecular Ion M+ (m/z)	Retention Time (minutes)	White-fleshed Potato Chips	Purple-fleshed Potato Chips
		Phenolic Acids		
P-coumaric acid	165.1	5.55	527.5 ± 41.9	544.5 ± 23.2
Chlorogenic acid	355.1	6.08	6543.5 ± 35.9	15176.6 ± 73.9
		Anthocyanins		
Pet-3-rut-5-glc	787.3	5.79	0.0	1578.6 ± 105.7
Mal-3-rut-5-glc	801.3	6.19	0.0	237.5 ± 14.8
Cya-3-O(6-O-malonyl-β-D-glc)	535.1	6.37	0.0	691.1 ± 3.2
Peo-3-(p-coum)-isophoro-5-glc	933.3	7.26	0.0	2729.2 ± 275.7
Peo-3-rut-5-glc	771.3	7.80	0.0	2871.1 ± 29.9
Pet-3-(p-coum)-rut-5-glc	933.3	7.92	0.0	28748.5 ± 235.7
Peo-3-caffeyl-rut-5-glc	933.3	8.03	0.0	27215.4 ± 2295.1
Pel-3-(p-coum)-rut-5-glc	887.3	8.11	0.0	80.8 ± 5.4
Pel-3-(4'''-ferul-rut)-5-glc	917.3	8.15	0.0	1569.3 ± 142.7
Peo-3-(p-coum)-rut-5-glc	917.3	8.21	0.0	1810.2 ± 135.2
Mal-3-(p-coum)-rut-5-glc	947.3	8.31	0.0	2707.4 ± 204.4

The compounds are reported as the area under the curve per gram dry weight. Values are presented as the means ± S.E. of 6 replicates.

4.4.2 Physical Parameters

Initial body weight of pigs did not differ among treatments. There were no significant differences in overall feed intake and overall weight gain among four treatment groups at week 5 (C, P10, P20 and W10), however, W20 group had lower overall feed intake and weight gain compared to control ($P \leq 0.05$, Table 4.4). However, by week 5, there were no significant differences in body weight, weight gain or feed intake among the 5 treatment groups. It should be noted that, overall feed conversion ratio, weight gain/feed consumed, did not differ among the five groups of animals at the end of the study (0.3 ± 0.1 for all groups, Appendix I). No significant differences were observed among treatment groups in back-fat thickness (Appendix I). All the 40 animals completed the study, and no adverse side effects/infections were reported.

Table 4.4 Growth performances (body weight, weight gain and feed intake) of pigs consuming experimental diets during the 5 week feeding period.

Dietary Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	
			Body Weight (kg)				
C	134.5 ± 2.9[a]	140.1 ± 3.3[a]	145.8 ± 3.7[a]	151.0 ± 4.1[a]	153.6 ± 3.9[a]	158.6 ± 4.0[a]	
P10	133.6 ± 2.1[a]	139.7 ± 2.2[a]	143.6 ± 2.4[a]	150.0 ± 2.4[a]	154.0 ± 2.3[a]	157.7 ± 2.1[a]	
P20	133.2 ± 2.6[a]	133.9 ± 2.8[b]	139.8 ± 3.1[ab]	144.8 ± 2.4[a]	151.6 ± 3.2[a]	155.5 ± 3.5[a]	
W10	132.3 ± 3.0[a]	137.6 ± 3.2[ab]	141.2 ± 3.6[ab]	146.4 ± 3.1[a]	150.1 ± 3.1[a]	153.8 ± 3.2[a]	
W20	132.8 ± 2.8[a]	132.4 ± 3.5[b]	134.6 ± 4.3[b]	143.6 ± 4.2[a]	147.1 ± 3.7[a]	150.0 ± 3.4[a]	
			Average Daily Gain (kg/d)				**Overall Weight Gain (kg/d)**
C		0.8 ± 0.1[a]	0.8 ± 0.1[a]	0.7 ± 0.1[a]	0.4 ± 0.1[a]	0.7 ± 0.1[a]	0.7 ± 0.0[a]
P10		0.9 ± 0.1[a]	0.6 ± 0.1[ab]	0.9 ± 0.1[a]	0.6 ± 0.1[ab]	0.5 ± 0.1[a]	0.7 ± 0.0[a]
P20		0.1 ± 0.3[b]	0.8 ± 0.1[a]	0.7 ± 0.2[a]	1.0 ± 0.2[b]	0.6 ± 0.1[a]	0.6 ± 0.0[ab]
W10		0.8 ± 0.1[a]	0.5 ± 0.1[ab]	0.7 ± 0.1[a]	0.5 ± 0.1[a]	0.5 ± 0.1[a]	0.6 ± 0.0[ab]
W20		-0.1 ± 0.3[b]	0.3 ± 0.2[b]	1.3 ± 0.2[b]	0.5 ± 0.2[a]	0.4 ± 0.1[a]	0.5 ± 0.1[b]

	Average Daily Feed Intake (kg/d)					Overall Feed Consumed (kg/d)
C	2.8 ± 0.1[a]	2.4 ± 0.1[a]	2.6 ± 0.1[a]	2.1 ± 0.1[a]	1.9 ± 0.1[a]	2.4 ± 0.1[a]
P10	2.5 ± 0.2[a]	2.2 ± 0.1[ab]	2.6 ± 0.1[a]	2.2 ± 0.1[a]	2.0 ± 0.1[a]	2.3 ± 0.1[a]
P20	1.3 ± 0.3[b]	2.1 ± 0.2[ab]	2.6 ± 0.1[a]	2.5 ± 0.2[a]	2.1 ± 0.1[a]	2.1 ± 0.1[ab]
W10	2.1 ± 0.3[a]	2.0 ± 0.1[ab]	2.4 ± 0.1[a]	2.0 ± 0.1[a]	1.7 ± 0.1[a]	2.1 ± 0.1[ab]
W20	1.1 ± 0.3[b]	1.8 ± 0.3[b]	2.3 ± 0.2[a]	2.1 ± 0.1[a]	1.9 ± 0.1[a]	1.8 ± 0.1[b]

Data are presented as means ± S.E. Means in columns (within a week between treatment groups) that differ by a common letter (a, b) differ ($P \leq 0.05$). C: High-fat diet control, P10 and P20 - purple-fleshed potato chips at 10 and 20 % of diet w/w and W10 and W20 - white-fleshed potato chips at 10 and 20 % of the diet w/w.

4.4.3 Colonic GSH:GSSG Ratio

Glutathione is an intracellular anti-oxidant in cells. Glutathione exists in two forms reduced (GSH) and oxidized (GSSG). When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSH to GSSG decreases. Therefore, the determination of the GSH:GSSG ratio is a useful indicator of oxidative stress in animal tissues (Owen and Butterfield 2010). We measured GSH:GSSG ratio in the distal colon of the animals. The animals consuming HFD for the entire duration of the study (C) had lower GSH:GSSG ratio compared to animals in the potato groups. GSH:GSSG ratio was significantly higher only in the animals consuming purple-fleshed potato chips at 10 % (P10) compared to the HFD control (P = 0.05, Figure 4.2). This suggests that the color of the potato and the supplementation levels are important considerations to suppress HFD induced oxidative stress indexed by glutathione redox ratio in the distal colon.

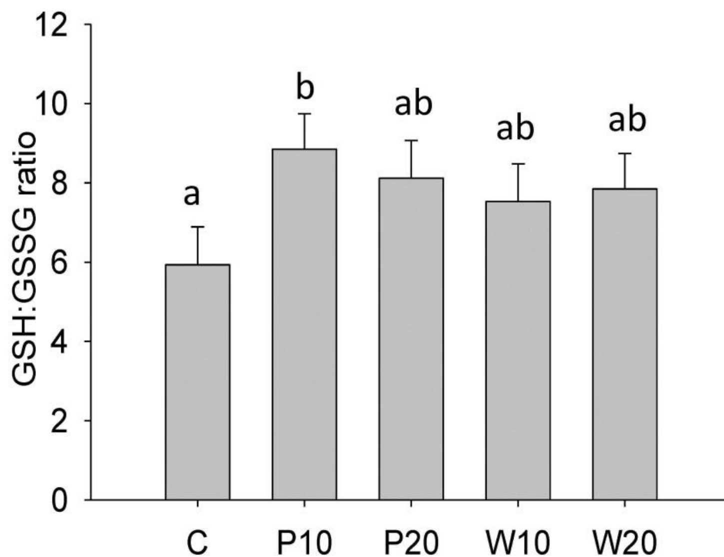

Figure 4.2 Glutathione (GSH-reduced and GSSG-oxidized) were measured in the distal colon using UPLC/PDA. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control,

letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

4.4.4 Colonic Inflammatory Markers

We measured expression of a panel of innate inflammatory markers in distal colon mucosa using qPCR. Relative expression of the pro-inflammatory markers including NF-κB, TNF-α, TLRs 2 and 4 were suppressed, irrespective of dosage, only in the purple-fleshed potato diet groups compared to the animals on HFD control (NF-κB, $P = 0.05$; TNF-α, $P = 0.05$; TLR-2, $P = 0.02$; and TLR-4, $P = 0.02$). The relative expression of TLR-2 and TLR-4 in the white-fleshed potato diet group were not statistically significant compared to HFD control or the purple-fleshed potato diet group (Figures 4.3 A-C). We measured relative expression of anti-inflammatory cytokines IL-10 and TGF-β in the distal colon mucosa of these animals. There were no significant differences among groups (Figure 4.3 D).

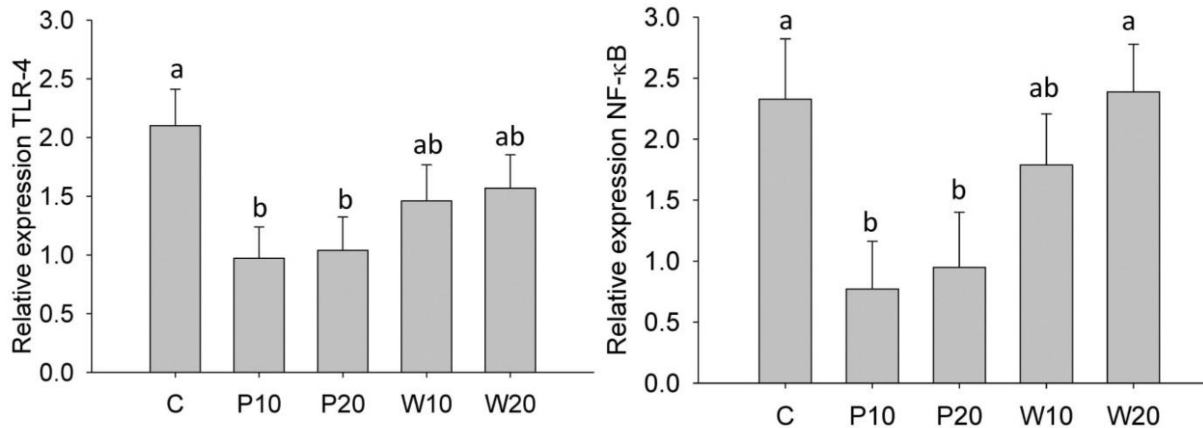

Figure 4.3A Relative expression of TLR-4 and NF-κB as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control,

letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

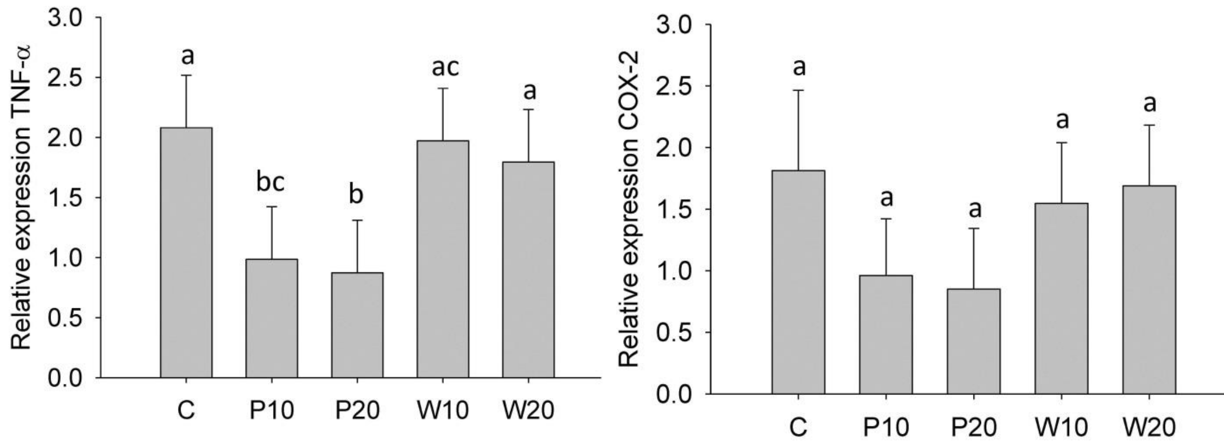

Figure 4.3B Relative expression of TNF-α and COX-2 as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

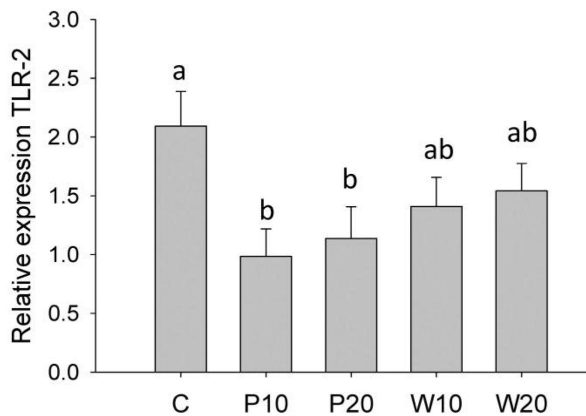

Figure 4.3C Relative expression of TLR-2 as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W

indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

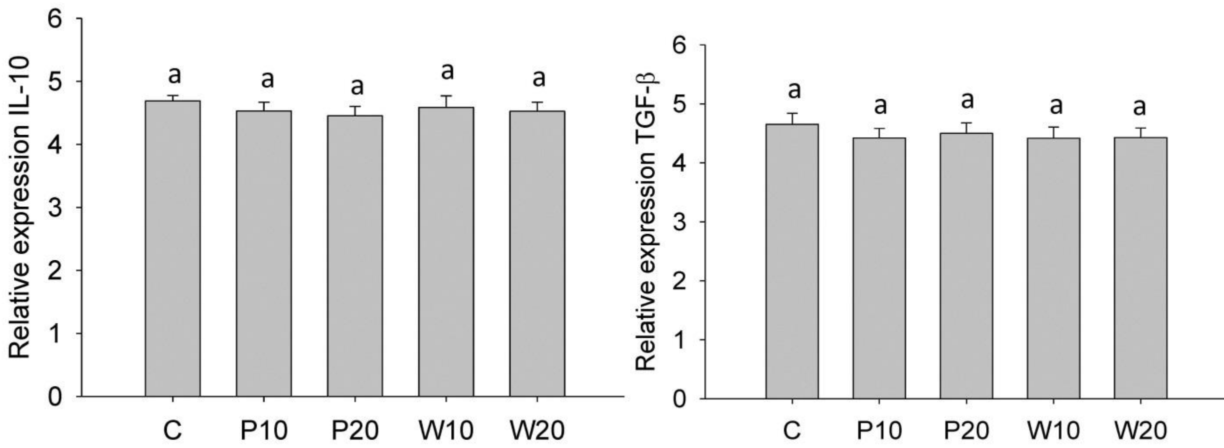

Figure 4.3D Relative expression of IL-10 and TGF-β as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

4.4.5 Mesenteric Fat GSH:GSSG Ratio

As a marker for oxidative stress, we measured GSH:GSSG ratio in the mesenteric fat of the animals. Only the purple-fleshed potato consuming animals, P10 group, tended to have higher mesenteric fat GSH:GSSG ratio compared to the HFD controls ($P = 0.1$). GSH:GSSG ratio in animals in the white-fleshed potato groups were not significantly different compared to animals on HFD control or the purple-fleshed potato group P20 (Figure 4.4).

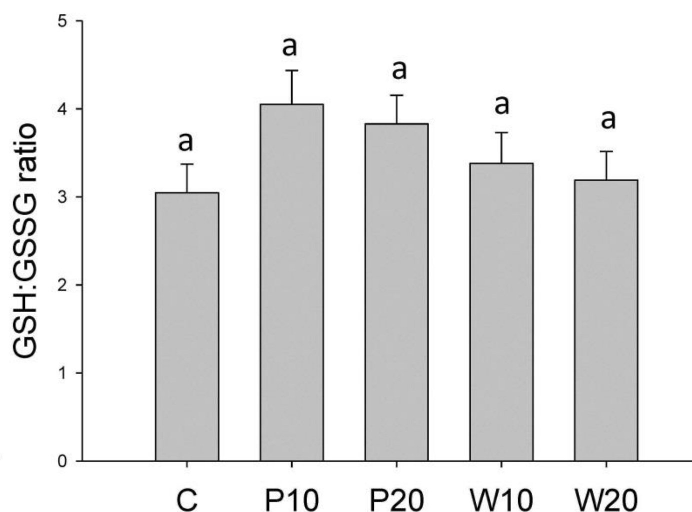

Figure 4.4 Glutathione (GSH-reduced and GSSG-oxidized) were measured in the mesenteric fat using UPLC/PDA. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

4.4.6 Mesenteric Fat Inflammatory Markers

We measured relative expression of the innate inflammatory markers in the mesenteric fat. Only the animals consuming purple-fleshed potato (P10 and P20) had suppressed TNF-α ($P = 0.05$) and numerically lower NF-κB and TLR-4 ($P \leq 0.15$) expression in the mesenteric fat compared to the HFD control animals (Figure 4.5 A, 4.5 B). Mesenteric fat expression of TNF-α, NF-κB and TLR-4 in the animals consuming white-fleshed potato diet W10 was similar to the purple-fleshed potato groups (Figure 4.5 A, 4.5 B).

Expression of adiponectin, an anti-inflammatory adipokine and an insulin sensitizer, was elevated in the purple-fleshed potato consuming animals (P10) compared to animals on the HFD

control and the white-fleshed potato (Figure 4.5 A, $P \leq 0.05$). In addition, expression of anti-inflammatory cytokines IL-10 and TGF-β were higher in the animals consuming purple-fleshed potato P10 compared to the animals on the white-fleshed potato diets, however, were not significantly different from HFD control group (Figure 4.5 C, $P \leq 0.05$).

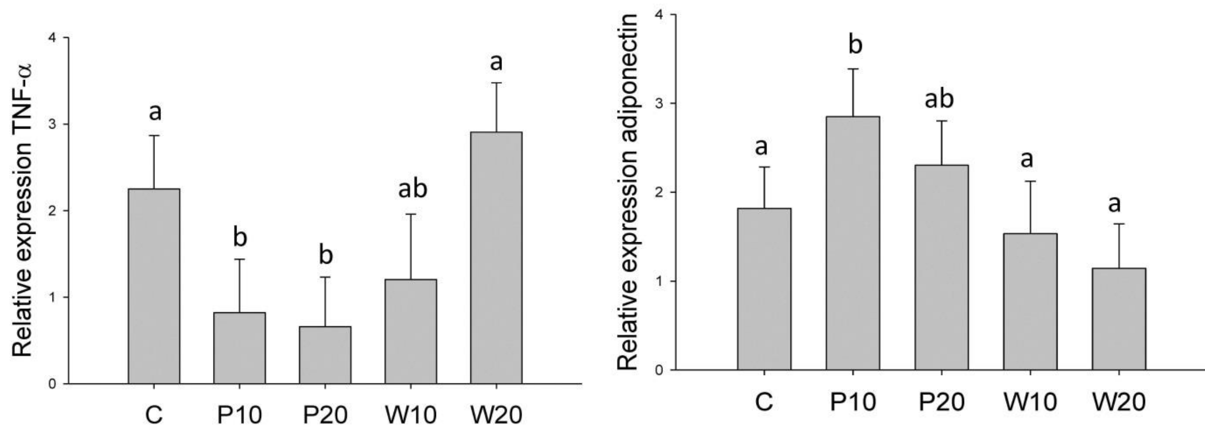

Figure 4.5A Relative expression of TNF-α and adiponectin as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

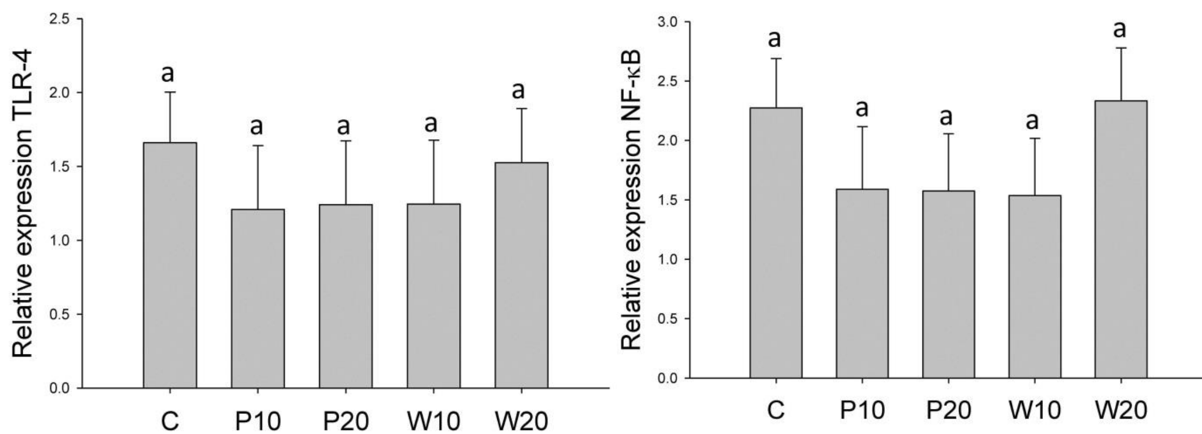

Figure 4.5B Relative expression of TLR-4 and NF-κB as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group.

Means that differ by a common letter (a, b) differ (P ≤ 0.05). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

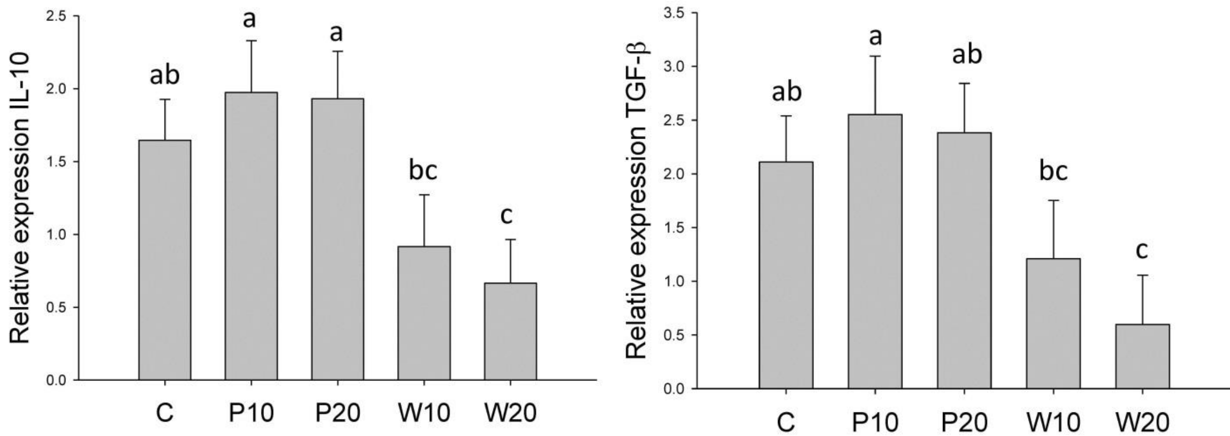

Figure 4.5C Relative expression of IL-10 and TGF-β as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b, c) differ (P ≤ 0.05). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

4.4.7 Systemic Oxidative Stress Markers

Serum 8-isoprostane (8IP) concentrations (pg/ml) were lower in both purple-fleshed potato diet (P10; P ≤ 0.03, P20; P ≤ 0.04) consuming groups compared to HFD control (Figure 4.6). Serum concentration (μmol/L) of MDA, a lipid peroxidation marker, tended to be suppressed by the P10 diet compared to HFD control (Figure 4.6, P ≤ 0.07 for P10). There was a significant correlation between MDA and 8IP (P ≤ 0.03; r = 0.54). However, there were no significant differences in urinary concentrations of 8IP or DNA adduct 8-hydroxy-2′-

deoxyguanosine among the treatment groups (Appendix II). Potato consumption did not significantly elevate serum total anti-oxidant status (Appendix II).

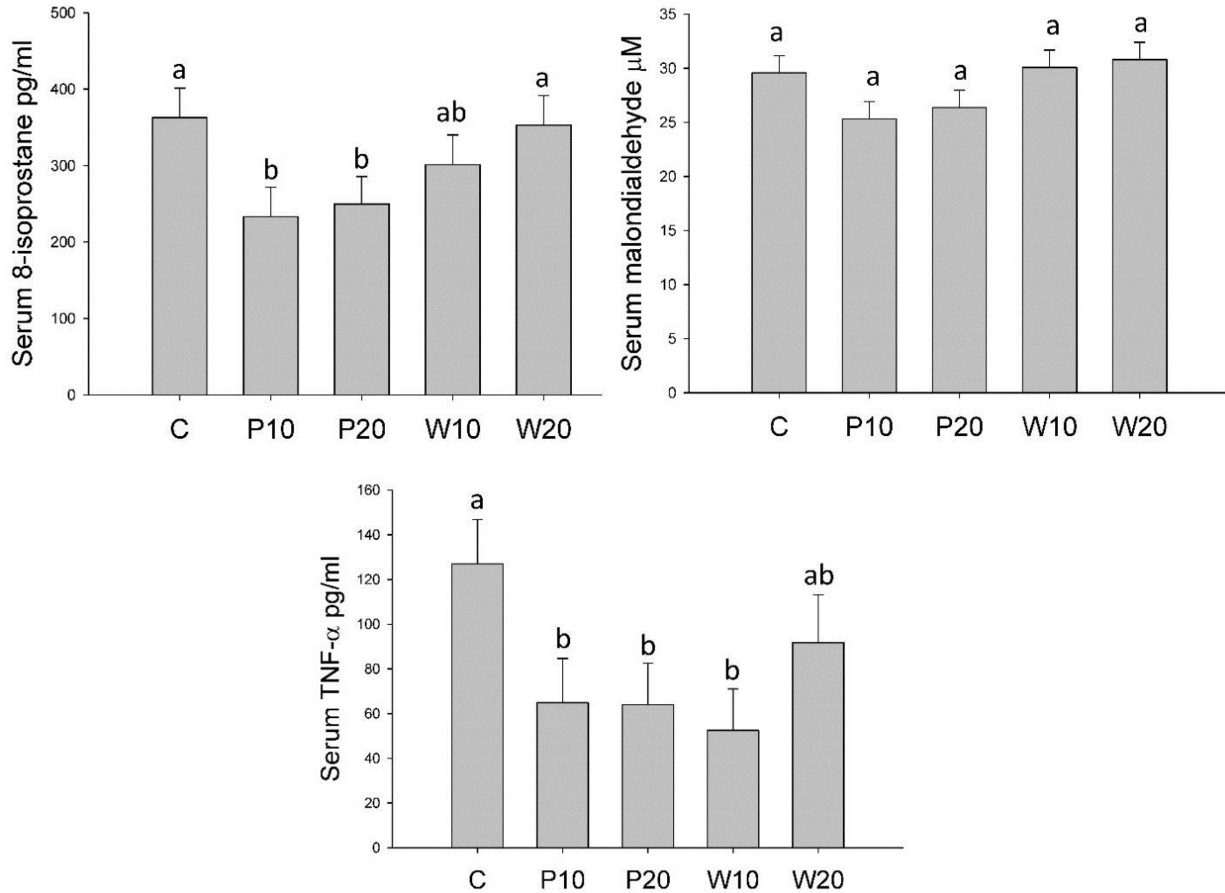

Figure 4.6 8-isoprostane was measured using ELISA following manufacturers protocol (Cayman). Malondialdehyde was measured using a colorimetric TBARS assay (Cayman). TNF-α was measured in porcine serum using ELISA following manufacturers protocol (Life Technologies). Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C, high-fat diet control, letters P and W indicate the chipped purple and white-fleshed potato diets and the numbers 10 and 20 indicate 10 and 20 % potato supplementation in the diet (w/w).

4.4.8 Serum Inflammatory Markers

Serum TNF-α concentration was lower in purple-fleshed potato diet ($P \leq 0.05$) consuming animals compared to HFD control. Even the white-fleshed potato consuming animals, W10, had lower TNF-α concentration ($P \leq 0.05$) compared to HFD control animals (Figure 4.6). However, there were no significant differences among treatments in serum concentrations of other inflammatory markers including C-reactive protein, lipopolysaccharide, and the eicosanoid LTB4 (Appendix II) among the treatment groups.

4.5 Discussion

Our results demonstrate that processed purple-fleshed potato supplementation in the diet of the HFD consuming animals reversed the elevated oxidative stress and inflammation biomarkers in the distal colon, mesenteric fat and systemic circulation, compared to HFD controls.

We measured the differences in the phenolic profile between white and purple-fleshed potato chips using UPLC/MS. In the volcano plot (Figure 4.1B), over 400 features were higher in purple-fleshed potato compared to white-fleshed potato, whereas only 62 features were higher in the white-fleshed potato (P value ≤ 0.01, fold change > 4). This shows that the white-fleshed potatoes have certain phenolic compounds in higher abundance over the purple-fleshed cultivars although the number is significantly less. Moreover, from table 4.1, it is evident that both purple- and white-fleshed potatoes retained phenolic acids post processing into chips. Anthocyanins, however, were only present in the purple-fleshed cultivars.

4.5.1 Purple-fleshed Potato Reversed Colonic and Mesenteric Fat Oxidative Stress (GSH:GSSG Ratio) in HFD Consuming Animals.

The GSH and GSH disulfide (GSSG) systems play a critical role in determining intracellular redox balance and antioxidant function. GSH has a central role as a tissue antioxidant and its oxidative product GSSG, is reduced back to GSH by GSSG reductase. GSH in the gut lumen is required for normal intestinal function because its antioxidant functions protect the lumen from damage by ROS (Benton, Liang et al. 2012). Depletion of GSH can lead to degeneration of colonic epithelial cells in mice and is also associated with upregulated levels of pro-inflammatory cytokines (Haddad 2011). Indeed, studies in animal models showed that intestinal ischemia, radiation injury, or infection is associated with a decrease in GSH in tissues and associated organ dysfunction (Holmes, Yong et al. 1998, Benton, Liang et al. 2012). Considering the apparent role of oxidative stress in inflammatory conditions of the colon, and the importance of GSH to the integrity of the colon (Holmes, Yong et al. 1998), we measured both GSH and GSSG in the distal colon using a highly sensitive UPLC/PDA method (Yilmaz, Keser et al. 2009). Our results demonstrate that only the consumption of purple-fleshed potato (P10) suppressed distal colonic oxidative stress as measured using GSH:GSSG ratio compared to HFD control (Figure 4.2).

The adipose tissue is subjected to increased oxidative stress (Berg and Scherer 2005) in HFD consuming animal models (Charradi, Elkahoui et al. 2013). Elevated ROS seen in hypertrophied adipose tissue is accompanied by a decrease in mRNA expression levels and activities of antioxidant enzymes such as glutathione peroxidase (GPX), Cu/Zn superoxide dismutase (Cu/Zn SOD), and catalase, which are essential for homeostasis of the redox state and are induced to detoxify ROS when cells are exposed to oxidative stress. GSH is an essential

factor for the enzymatic function of GPXs on the reduction reaction and also acts as an antioxidant itself to scavenge ROS. The regulatory systems of intracellular GSH content and GSH/GSSG ratio are not well known in the adipose tissue, however as GSH itself is an antioxidant, accumulation of GSH will protect against ROS (Kobayashi, Matsuda et al. 2009). Our results suggest that in the mesenteric fat, the animals consuming purple-fleshed potato group P10 tended to have elevated GSH:GSSG ratio compared to the HFD controls (P = 0.1, Figure 4.4). Thus, unlike the distal colon, in the mesenteric fat, purple-fleshed potato consumption provides only a modest anti-oxidant benefit as measured using GSH:GSSG ratio in HFD consuming pigs.

4.5.2 Purple-fleshed Potato Reversed Colonic and Mesenteric Fat Inflammatory Markers in HFD Consuming Animals.

HFD has shown to cause changes in the gut bacterial population – leading to elevated lipopolysaccharide (LPS) induced toll like receptor-4 (TLR4) activation in the colon (Li, Lelliott et al. 2008). Saturated fatty acids, typically present in Western diets, can activate TLRs directly and leading to innate inflammatory response not only in the colon, but also in the adipose tissue (Chait and Kim 2010, Maury and Brichard 2010). TLR activation is a key element for induction of the innate inflammatory response via triggering downstream signaling cascades including the transcription factor NF-κB (Takeda and Akira 2004). Indeed, studies have shown elevated expression of both TLR-2 and TLR-4 in the colonic mucosa of children with inflammatory bowel disease (Szebeni, Veres et al. 2008); mice lacking TLR-4 were protected from HFD induced colonic inflammation and obesity (Kim, Gu et al. 2012). Thus, we measured expression of TLR-2 and TLR-4 in the colonic mucosa in these animals.

In our study, only the purple-fleshed potato consuming animals had lower relative expression of TLR-2 and TLR-4 compared to the HFD control. There were no significant differences among the white-fleshed potato groups and the HFD controls (Figure 4.3 A, C). We measured relative expression of TLR-4 in the proximal colon and ileum mucosa of these animals in both studies. We did not observe any significant differences in the relative expression of TLR-4 among the treatment groups (Appendix III). In addition, we did not see any significant alterations in the TLR-4 expression in the mesenteric fat (Figure 4.5 B).

We measured relative expression of a broad panel of innate inflammatory mediators in the distal colon mucosa and mesenteric fat of these animals. Only the animals consuming purple-fleshed potato supplemented diets had significantly lower distal colonic expression of NF-κB and TNF-α compared to the HFD controls. Levels in the white-fleshed potato group did not differ significantly compared to the HFD control (Figure 4.3 A-C). This is in concert with earlier studies in colitis models, where anthocyanins have shown amelioration of experimental colitis (Piberger, Oehme et al. 2011, Wu, Xu et al. 2011). In particular, bilberry anthocyanins (Piberger, Oehme et al. 2011) in mice suppressed intestinal inflammation in acute and chronic DSS-colitis with significant suppression of TNF-α and IFN-γ.

Since under physiological normal conditions a balance is maintained between pro- and anti-inflammatory indices, we measured relative expression of anti-inflammatory cytokines IL-10 and TGF-β in the distal colon mucosa of these animals. There were no differences in the relative expression of anti-inflammatory cytokines; TGF-β and IL-10, among the treatment groups in the distal colon mucosa (Figure 4.3 D). Results suggest that the consumption of purple-fleshed potatoes reversed elevated distal colonic inflammatory markers in HFD consuming animals. White-fleshed potato consumption, in the short duration of the study could not reverse

the detrimental effects of HFD. We measured expression of NF-κB and TNF-α in the proximal colon and ileum mucosa of these animals. We did not detect any significant difference among the animals consuming potato diets compared to the HFD control (Appendix III).

Most adipokines with pro-inflammatory properties (e.g. TNF-α) are overproduced with increasing adiposity, while some anti-inflammatory adipokines like adiponectin are decreased (Berg and Scherer 2005, Maury and Brichard 2010). This dysregulation of adipokine production may promote risk for chronic diseases (Berg and Scherer 2005). Our results show that, the purple-fleshed potato consuming animals had lower mesenteric fat expression of TNF-α, a critical pro-inflammatory cytokine (Figure 4.5 A). In addition, expression of adiponectin was elevated in the P10 group compared to the HFD controls and the white-fleshed potato groups (Figure 4.5 A). However, there were no significant differences in NF-κB expression in the mesenteric fat (Figure 4.5 B). Results demonstrate only a marginal beneficial effect of purple-fleshed potato consumption on oxidative stress and inflammatory markers in the mesenteric fat.

We measured expression of IL-10 and TGF-β in the mesenteric fat of these animals. The white-fleshed potato groups had the numerically lowest expression levels even when compared to the HFD controls. The animals in the P10 group had significantly elevated IL-10 and TGF-β expression compared to the white-fleshed potato consuming groups, however, were not different from the HFD controls (Figure 4.5 C).

4.5.3 Purple-fleshed Potato Reversed Systemic Oxidative Stress Markers in HFD Consuming Animals.

F_2 isoprostanes are prostaglandin-F_2-like compounds formed by oxidation of arachidonic acid in the cell membrane via non-enzymatic (COX-2 independent) pathway. Concentrations of

8-isoprostane, one of the more prevalent F_2 isoprostanes, in the plasma/serum and urine are regarded as one of the best indices of lipid peroxidation, and oxidative stress that are currently available (Montuschi, Barnes et al. 2004). Systemic 8-isoprostane concentrations are elevated in high-fat diet fed mice (Sandu, Song et al. 2005), and is an important oxidative stress biomarker in chronic diseases (Schwedhelm, Bartling et al. 2004, Kaviarasan, Muniandy et al. 2009).

In our study, we measured both free and bound 8-isoprostane in the serum, and urine of the HFD consuming pigs. Both the purple-fleshed potato diets suppressed serum 8-isoprostane concentrations compared to HFD control. Serum concentrations of 8-isoprostane in white-fleshed potato group were not significantly different from HFD control suggesting benefits of purple-fleshed potato consumption (Figure 4.6). However, no significant differences were seen in urinary 8-isoprostane concentrations among the treatments.

To confirm suppression of oxidative stress, we measured MDA, which is an oxidative stress biomarker in systemic circulation and in tissue, used for over 30 years (Lykkesfeldt 2007). MDA is mainly produced in biological samples via peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds or by enzymatic processes from various prostaglandins (Del Rio, Stewart et al. 2005). MDA is an important marker to measure systemic oxidative stress and is used in many studies employing dietary interventions in high-fat diet consuming animal models (Meng, Zhu et al. 2011, He, Wang et al. 2012). In our study, there was only a tendency for MDA to be suppressed in the animals consuming purple-fleshed potato diet (P10), Figure 4.6). There were no differences among HFD control and the white-fleshed potato diets consuming animals. Significant correlation observed between MDA and 8IP ($P \leq 0.03$; $r = 0.54$) lends support to the anti-oxidant benefits of purple-fleshed potato consumption.

4.5.4 Purple-fleshed Potato Suppressed Serum TNF-α in HFD Consuming Animals.

TNF-α is a critical pro-inflammatory cytokine and elevated levels of TNF-α in the systemic circulation are linked to variety of disorders. TNF-α is linked to heart disease, as it works with other pro-inflammatory cytokines, chemokines, and various immune cells towards the development of atherosclerotic lesions, the recruitment and activation of inflammatory cells, and the initiation of the inflammatory cascade inside the arterial wall. TNF-α also contributes to endothelial dysfunction in ischemia/reperfusion injury (Zhang, Xu et al. 2006, Gao, Xu et al. 2007). TNF-α level in the blood were found to positively correlate with markers of insulin resistance in a community-based cohort study (Hivert, Sullivan et al. 2008, Ouchi, Parker et al. 2011). Blocking TNF- α in mice reduced colorectal carcinogenesis associated with chronic colitis (Popivanova, Kitamura et al. 2008) – thus, TNF-α is an important target for anti-inflammatory interventions against chronic diseases.

The purple-fleshed potato groups (P10 and P20) and the white-fleshed potato group (W10) had lower concentrations of TNF-α compared to HFD control (Figure 4.6). Interestingly, similar expression profiles of TNF-α, and NF-κB and TLR-4 (non-significant, tends only) were seen in the mesenteric fat of these animals among the treatment groups (Figure 4.5 A-B). Although white-fleshed potatoes lack anthocyanins, they contain phenolic acids such as chlorogenic acid, caffeic acid, p-coumaric acid etc. that demonstrated anti-inflammatory properties in multiple studies (da Cunha, Duma et al. 2004, Chao, Mong et al. 2010, Chauhan, Satti et al. 2011). Indeed, we found 62 features that were higher and significantly different (fold change > 4 and $P \leq 0.01$) in white-fleshed potato compared to purple-fleshed potato (Figure 4.1B). Phenolics acids like caffeic acid, rich in white-fleshed potatoes, have shown TNF-α lowering ability *in vivo* (Chao, Hsu et al. 2009). In addition, potatoes in general are rich sources

of Vitamin C, resistant starch and their contribution cannot be neglected. However, to elucidate the contribution of different bioactive compounds in the potato towards the *in vivo* anti-inflammatory activity, further research is necessary. Our results, however, provide first *in vivo* evidence that purple-fleshed potatoes, even after processing, retain their anti-oxidant and anti-inflammatory properties.

4.5.5 Limitations of the Study

Although, white-fleshed potatoes could not reverse the HFD elevated oxidative stress and inflammatory biomarkers in the distal colon, mesenteric fat and systemic circulation, there was a trend for beneficial effects of consuming white-fleshed potato. This study was short (5 weeks) and might explain the difference. However, purple-fleshed potato consumption demonstrated beneficial effects in the short time period suggesting the potent anti-inflammatory and anti-oxidant efficacy of anthocyanins and higher phenolic acids present in the purple-fleshed potato.

One limitation of the study is the lack of normal control animals to elucidate deleterious effect of consumption of a HFD. The study mentioned in ((Radhakrishnan, Reddivari et al. 2012), Chapter V) employed a standard control, and we showed that standard control animals had lower systemic 8-isoprostane and TNF-α compared to pigs on a HFD. Another limitation of the study is the relatively high dose of potato chips (10-20 %) of diet; on an average the animals consume approximately 1.2-3 kg of chips per week. However, the potato is an important staple food for various populations around the world and it provides approximately 5 % to 15 % of dietary calories (Thompson, Thompson et al. 2009). It should be noted that over 80 % of potato is consumed in processed form, mainly frying (NPC 2005, NPC 2007, NPC 2010).

It is interesting to note that in the present study, we found the 10 % purple-fleshed potato group was similar and in some cases more effective in modulating oxidative stress and inflammatory parameters than the 20 % dose. The phenomenon that lower doses can be equal or more effective than the higher doses has been observed with other phenolic compounds. For e.g., freeze dried mango supplementation in the HFD in mice at 1 % supplementation was more effective in improving glucose tolerance and lipid profile and reducing adiposity associated with a HF diet compared to 10 % supplementation (Lucas, Li et al. 2011). Moreover, a study by Marugundanan et al. (Muruganandan, Srinivasan et al. 2005) demonstrated that mangiferin at 10 mg/kg is as effective as the 20 mg/kg dose for its anti-diabetic, anti-hyperlipidaemic and anti-atherogenic activities in streptozotocin-diabetic rats.

Moreover, potatoes are known to contain toxicants such as glycoalkaloids. As the potatoes were processed into chips, higher dosage of chips elevates consumption of processing induced toxin – acrylamide which is known to induce inflammation and is carcinogenic (Capuano and Fogliano 2011, Zhang, Wang et al. 2013). Thus, higher consumption of potato chips elevates the concentrations of these toxicants which might counter the health benefits of the bioactive compounds in the potato. Thus, we need to identify the optimal dose of foods and its bioactive compounds that exert a health benefit and not to work under the assumption that more is better.

4.6 Conclusions

Studies in mice have shown that mucosal inflammation leads to systemic genotoxicity characterized by elevated systemic oxidative stress markers, DNA damage and inflammatory cytokines and contributes early on to genetic instability necessary for progression to inflammatory bowel disease-associated dysplasia and the development of cancer (Westbrook, Wei et al. 2009). Results of our study suggests that chipped purple-fleshed potatoes suppress oxidative stress and inflammatory markers in the distal colon, mesenteric fat, and systemic circulation and thus, may suppress risk for colon cancer and other chronic disorders linked with HFD. Due to high consumption of potatoes, purple-fleshed potatoes can be an effective delivery vehicle for health promoting bioactive compounds.

4.7 References

Apel, K. and H. Hirt (2004). "Reactive oxygen species: metabolism, oxidative stress, and signal transduction." Annu Rev Plant Biol **55**: 373-399.

Arkan, M. C., A. L. Hevener, F. R. Greten, S. Maeda, Z. W. Li, J. M. Long, A. Wynshaw-Boris, G. Poli, J. Olefsky and M. Karin (2005). "IKK-beta links inflammation to obesity-induced insulin resistance." Nat Med **11**(2): 191-198.

Bastard, J. P., M. Maachi, C. Lagathu, M. J. Kim, M. Caron, H. Vidal, J. Capeau and B. Feve (2006). "Recent advances in the relationship between obesity, inflammation, and insulin resistance." Eur Cytokine Netw **17**(1): 4-12.

Benton, S. M., Z. Liang, L. Hao, Y. Liang, G. Hebbar, D. P. Jones, C. M. Coopersmith and T. R. Ziegler (2012). "Differential regulation of tissue thiol-disulfide redox status in a murine model of peritonitis." J Inflamm **9**(1): 36.

Berg, A. H. and P. E. Scherer (2005). "Adipose tissue, inflammation, and cardiovascular disease." Circ Res **96**(9): 939-949.

Brown, C. R. (2005). "Antioxidants in potato." Am J Potato Res **82**: 163-172.

Capuano, E. and V. Fogliano (2011). "Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies." LWT-Food Sci Tech **44**(4): 793-810.

Chait, A. and F. Kim (2010). "Saturated fatty acids and inflammation: who pays the toll?" Arterioscler Thromb Vasc Biol **30**(4): 692-693.

Chao, C. Y., M. C. Mong, K. C. Chan and M. C. Yin (2010). "Anti-glycative and anti-inflammatory effects of caffeic acid and ellagic acid in kidney of diabetic mice." Mol Nutr Food Res **54**(3): 388-395.

Chao, P. C., C. C. Hsu and M. C. Yin (2009). "Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice." Nutr Metab (Lond) **6**: 33.

Charradi, K., S. Elkahoui, F. Limam and E. Aouani (2013). "High-fat diet induced an oxidative stress in white adipose tissue and disturbed plasma transition metals in rat: prevention by grape seed and skin extract." J Physiol Sci **63**(6): 445-455.

Chauhan, P. S., N. K. Satti, V. K. Sharma, P. Dutt, K. A. Suri and S. Bani (2011). "Amelioration of inflammatory responses by Chlorogenic acid via suppression of pro-inflammatory mediators." J App Pharma Sci **1**(4): 67-75.

Chuang, C. C. and M. K. McIntosh (2011). "Potential mechanisms by which polyphenol-rich grapes prevent obesity-mediated inflammation and metabolic diseases." Annu Rev Nutr **31**: 155-176.

Chun, O., D. Kim, N. Smith, D. Schroeder, J. Han and C. Lee (2005). "Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet." J Sci Food Agric **85**(10): 1715-1724.

Cooper, D. A., D. A. Berry, V. A. Spendel, A. L. Kiorpes and J. C. Peters (1997). "The domestic pig as a model for evaluating olestra's nutritional effects." J Nutr **127**(8 Suppl): 1555S-1565S.

da Cunha, F. M., D. Duma, J. Assreuy, F. C. Buzzi, R. Niero, M. M. Campos and J. B. Calixto (2004). "Caffeic acid derivatives: in vitro and in vivo anti-inflammatory properties." Free Radic Res **38**(11): 1241-1253.

Del Rio, D., A. J. Stewart and N. Pellegrini (2005). "A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress." Nutr Metab Cardiovasc Dis **15**(4): 316-328.

Ding, S., M. M. Chi, B. P. Scull, R. Rigby, N. M. Schwerbrock, S. Magness, C. Jobin and P. K. Lund (2010). "High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse." PLoS One **5**(8): e12191.

Drouet, M., L. Dubuquoy, P. Desreumaux and B. Bertin (2012). "Visceral fat and gut inflammation." Nutrition **28**(2): 113-117.

Erdelyi, I., N. Levenkova, E. Y. Lin, J. T. Pinto, M. Lipkin, F. W. Quimby and P. R. Holt (2009). "Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer." J Nutr **139**(11): 2072-2078.

Erdelyi, I., N. Levenkova, E. Y. Lin, J. T. Pinto, M. Lipkin, F. W. Quimby and P. R. Holt (2009). "Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer." J Nutr. **139**(11): 2072-2078.

Gambero, A., M. Marostica, M. J. Abdalla Saad and J. Pedrazzoli, Jr. (2007). "Mesenteric adipose tissue alterations resulting from experimental reactivated colitis." Inflamm Bowel Dis **13**(11): 1357-1364.

Gao, X., X. Xu, S. Belmadani, Y. Park, Z. Tang, A. M. Feldman, W. M. Chilian and C. Zhang (2007). "TNF-alpha contributes to endothelial dysfunction by upregulating arginase in ischemia/reperfusion injury." Arterioscler Thromb Vasc Biol **27**(6): 1269-1275.

Haddad, J. J. (2011). "A redox microenvironment is essential for MAPK-dependent secretion of pro-inflammatory cytokines: modulation by glutathione (GSH/GSSG) biosynthesis and equilibrium in the alveolar epithelium." Cell Immunol **270**(1): 53-61.

He, H. J., G. Y. Wang, Y. Gao, W. H. Ling, Z. W. Yu and T. R. Jin (2012). "Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice." World J Diabetes **3**(5): 94-104.

Hivert, M. F., L. M. Sullivan, C. S. Fox, D. M. Nathan, R. B. D'Agostino, Sr., P. W. Wilson and J. B. Meigs (2008). "Associations of adiponectin, resistin, and tumor necrosis factor-alpha with insulin resistance." J Clin Endocrinol Metab **93**(8): 3165-3172.

Hogan, S., C. Canning, S. Sun, X. Sun and K. Zhou (2010). "Effects of Grape Pomace Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice." J Agric Food Chem **58**(21): 11250–11256.

Holmes, E. W., S. L. Yong, D. Eiznhamer and A. Keshavarzian (1998). "Glutathione content of colonic mucosa: evidence for oxidative damage in active ulcerative colitis." Dig Dis Sci **43**(5): 1088-1095.

Huang, D., B. Ou, M. Hampsch-Woodill, J. A. Flanagan and R. L. Prior (2002). "High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format." J Agric Food Chem **50**(16): 4437-4444.

Kaviarasan, S., S. Muniandy, R. Qvist and I. S. Ismail (2009). "F(2)-isoprostanes as novel biomarkers for type 2 diabetes: a review." J Clin Biochem Nutr **45**(1): 1-8.

Kim, K. A., W. Gu, I. A. Lee, E. H. Joh and D. H. Kim (2012). "High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway." PLoS One **7**(10): e47713.

Kobayashi, H., M. Matsuda, A. Fukuhara, R. Komuro and I. Shimomura (2009). "Dysregulated glutathione metabolism links to impaired insulin action in adipocytes." Am J Physiol Endocrinol Metab **296**(6): E1326-1334.

Lam, Y. Y., A. J. Mitchell, A. J. Holmes, G. S. Denyer, A. Gummesson, I. D. Caterson, N. H. Hunt and L. H. Storlien (2011). "Role of the gut in visceral fat inflammation and metabolic disorders." Obesity **19**(11): 2113-2120.

Li, H., C. Lelliott, P. Hakansson, K. Ploj, A. Tuneld, M. Verolin-Johansson, L. Benthem, B. Carlsson, L. Storlien and E. Michaelsson (2008). "Intestinal, adipose, and liver inflammation in diet-induced obese mice." Metabolism **57**(12): 1704-1710.

Lucas, E. A., W. Li, S. K. Peterson, A. Brown, S. Kuvibidila, P. Perkins-Veazie, S. L. Clarke and B. J. Smith (2011). "Mango modulates body fat and plasma glucose and lipids in mice fed a high-fat diet." Br J Nutr **106**(10): 1495-1505.

Lykkesfeldt, J. (2007). "Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking." Clin Chim Acta **380**(1-2): 50-58.

Madiwale, G. (2012). Effect of genotype, storage and processing on the polyphenolic content, composition, in vitro anti-cancer activity and sensory attributes of colored-flesh potatoes. Master of Science Thesis, Colorado State University.

Madiwale, G. P., L. Reddivari, D. G. Holm and J. Vanamala (2011). "Storage elevates phenolic content and antioxidant activity but suppresses antiproliferative and pro-apoptotic

properties of colored-flesh potatoes against human colon cancer cell lines." J Agric Food Chem **59**(15): 8155-8166.

Madiwale, G. P., L. Reddivari, M. Stone, D. G. Holm and J. Vanamala (2012). "Combined effects of storage and processing on the bioactive compounds and pro-apoptotic properties of color-fleshed potatoes in human colon cancer cells." J Agric Food Chem **60**(44): 11088-11096.

Matsuzawa-Nagata, N., T. Takamura, H. Ando, S. Nakamura, S. Kurita, H. Misu, T. Ota, M. Yokoyama, M. Honda, K. Miyamoto and S. Kaneko (2008). "Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity." Metabolism **57**(8): 1071-1077.

Maury, E. and S. M. Brichard (2010). "Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome." Mol Cell Endocrinol **314**(1): 1-16.

Meng, R., D. L. Zhu, Y. Bi, D. H. Yang and Y. P. Wang (2011). "Anti-Oxidative Effect of Apocynin on Insulin Resistance in High-Fat Diet Mice." Annals Clin Lab Sci **41**(3): 236-243.

Montuschi, P., P. J. Barnes and L. J. Roberts, 2nd (2004). "Isoprostanes: markers and mediators of oxidative stress." FASEB J **18**(15): 1791-1800.

Muruganandan, S., K. Srinivasan, S. Gupta, P. K. Gupta and J. Lal (2005). "Effect of mangiferin on hyperglycemia and atherogenicity in streptozotocin diabetic rats." J Ethnopharmacol **97**(3): 497-501.

NPC (2007). Potato statistical yearbook, National Potato Council (NPC).

NPC (2010). "Quick Fact About Potatoes." from http://www.nationalpotatocouncil.org/NPC/potato_quickfacts.cfm.

Ouchi, N., J. L. Parker, J. J. Lugus and K. Walsh (2011). "Adipokines in inflammation and metabolic disease." Nat Rev Immunol **11**(2): 85-97.

Owen, J. B. and D. A. Butterfield (2010). "Measurement of oxidized/reduced glutathione ratio." Methods Mol Biol **648**: 269-277.

Pan, M. H., C. S. Lai, S. Dushenkov and C. T. Ho (2009). "Modulation of inflammatory genes by natural dietary bioactive compounds." J Agric Food Chem. **57**(11): 4467-4477.

Piberger, H., A. Oehme, C. Hofmann, A. Dreiseitel, P. G. Sand, F. Obermeier, J. Schoelmerich, P. Schreier, G. Krammer and G. Rogler (2011). "Bilberries and their anthocyanins ameliorate experimental colitis." Mol Nutr Food Res **55**(11): 1724-1729.

Pond, W. G. and X. G. Lei (2000). Of pigs and people. Swine Nutrition. Washington, D.C., CRC Press**:** 3-18.

Popivanova, B. K., K. Kitamura, Y. Wu, T. Kondo, T. Kagaya, S. Kaneko, M. Oshima, C. Fujii and N. Mukaida (2008). "Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis." J Clin Investigation **118**(2): 560-570.

Pot, G. K., A. Geelen, G. Majsak-Newman, L. J. Harvey, F. M. Nagengast, B. J. Witteman, P. C. van de Meeberg, A. R. Hart, G. Schaafsma, E. K. Lund, G. T. Rijkers and E. Kampman (2010). "Increased consumption of fatty and lean fish reduces serum C-reactive protein concentrations but not inflammation markers in feces and in colonic biopsies." J Nutr **140**(2): 371-376.

Radhakrishnan, S., L. Reddivari, S. W. Kim and J. Vanamala (2012). "Uncooked and baked purple potatoes suppress oxidative stress and inflammatory markers in high-fat diet consuming pig." Potato Association of America - PAA 2012.

Radhakrishnan, S., L. Reddivari, G. Madiwale, S. W. Kim and J. Vanamala (2011). "Purple potato anthocyanins exert anti-cancer properties and suppress oxidative stress even after processing." Potato Association of America - PAA 2011.

Rodriguez-Saona, L. E., M. M. Giusti and R. E. Wrolstad (1998). "Anthocyanin pigment composition of red-fleshed potatoes." J Food Sci **63**(3): 458-465.

Romier-Crouzet, B., J. Van De Walle, A. During, A. Joly, C. Rousseau, O. Henry, Y. Larondelle and Y. J. Schneider (2009). "Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells." Food Chem Toxicol **47**(6): 1221-1230.

Rossi, A., I. Serraino, P. Dugo, R. Di Paola, L. Mondello, T. Genovese, D. Morabito, G. Dugo, L. Sautebin, A. P. Caputi and S. Cuzzocrea (2003). "Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation." Free Radic Res. **37**(8): 891-900.

Sandu, O., K. Song, W. Cai, F. Zheng, J. Uribarri and H. Vlassara (2005). "Insulin resistance and type 2 diabetes in high-fat-fed mice are linked to high glycotoxin intake." Diabetes **54**(8): 2314-2319.

Schwedhelm, E., A. Bartling, H. Lenzen, D. Tsikas, R. Maas, J. Brummer, F. M. Gutzki, J. Berger, J. C. Frolich and R. H. Boger (2004). "Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study." Circulation **109**(7): 843-848.

Shoelson, S. E., L. Herrero and A. Naaz (2007). "Obesity, inflammation, and insulin resistance." Gastroenterol **132**(6): 2169-2180.

Solinas, G. and M. Karin (2010). "JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction." FASEB J **24**(8): 2596-2611.

Szebeni, B., G. Veres, A. Dezsofi, K. Rusai, A. Vannay, M. Mraz, E. Majorova and A. Arato (2008). "Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease." Clin Exp Immunol **151**(1): 34-41.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol **16**(1): 3-9.

Thompson, M. D., H. J. Thompson, J. N. McGinley, E. S. Neil, D. K. Rush, D. G. Holm and C. Stushnoff (2009). "Functional food characteristics of potato cultivars (Solanum tuberosum L.): Phytochemical composition and inhibition of 1-methyl-1-nitrosourea induced breast cancer in rats." J Food Comp Anal **22**: 571-576.

Umesalma, S. and G. Sudhandiran (2010). "Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-kappaB, iNOS, COX-2, TNF-alpha, and IL-6 in 1,2-dimethylhydrazine-induced rat colon carcinogenesis." Basic Clin Pharmacol Toxicol **107**(2): 650-655.

Walton, M. C., R. G. Lentle, G. W. Reynolds, M. C. Kruger and T. K. McGhie (2006). "Anthocyanin absorption and antioxidant status in pigs." J Agric Food Chem **54**(20): 7940-7946.

Westbrook, A. M., B. Wei, J. Braun and R. H. Schiestl (2009). "Intestinal mucosal inflammation leads to systemic genotoxicity in mice." Cancer Res **69**(11): 4827-4834.

Wisse, B. E. (2004). "The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity." J Am Soc Nephrol **15**(11): 2792-2800.

Wu, L. H., Z. L. Xu, D. Dong, S. A. He and H. Yu (2011). "Protective Effect of Anthocyanins Extract from Blueberry on TNBS-Induced IBD Model of Mice." Evid Based Complement Alternat Med **2011**: 525462.

Yilmaz, Ö., S. Keser, M. Tuzcu, M. Güvenc, B. Çetintas, S. Irtegün, H. Tastan and K. Sahin (2009). "A Practical HPLC Method to Measure Reduced (GSH) and Oxidized (GSSG) Glutathione Concentrations in Animal Tissues." J Anim Vet Adv **8**(2): 343-347.

Zhang, C., X. Xu, B. J. Potter, W. Wang, L. Kuo, L. Michael, G. J. Bagby and W. M. Chilian (2006). "TNF-alpha contributes to endothelial dysfunction in ischemia/reperfusion injury." Arterioscler Thromb Vasc Biol **26**(3): 475-480.

Zhang, L. L., E. T. Wang, F. Chen, H. Y. Yana and Y. Yuan (2013). "Potential protective effects of oral administration of allicin on acrylamide-induced toxicity in male mice." Food Function **4**(8): 1229-1236.

CHAPTER V

PURPLE-FLESHED POTATOES PREVENTED HIGH-FAT DIET ELEVATED OXIDATIVE STRESS AND INNATE INFLAMMATION MARKERS IN A PIG MODEL.

5.1 Summary

Consumption of anthocyanins and anthocyanin-rich fruits has shown to suppress risk for colon inflammation, colon cancer, type 2 diabetes etc. Color-fleshed potato consumption increased by 17% in the US partly attributed to putative health benefits of bioactive constituents such as anthocyanins and phenolic acids. However, little information exists if purple-fleshed potatoes, rich in anti-oxidant and anti-inflammatory anthocyanins, even after processing, retain their health benefiting properties *in vivo*. We hypothesized that processed (raw vs. baked vs. chips) purple-fleshed potatoes will suppress high-fat diet (HFD) elevated oxidative stress/innate inflammation biomarkers in a young pig model. We performed a prevention study, where 64 pigs (n = 8/group), 3 weeks post-weaning, consumed one of the eight diets; standard control diet, HFD and HFD supplemented with purple or white-fleshed potato (raw, baked and chips, 10 % w/w) for 13 weeks. Potato diets had no effect on the average food intake and weight gain although the purple-fleshed potato baked group had numerically higher feed intake and weight gain compared to the other HFD groups. Irrespective of color/processing, pigs consuming potato diets had numerically lower oxidative stress (↑ GSH:GSSG ratio) and lower expression of inflammatory markers (NF-κB, $P = 0.04$; TNF-α, $P = 0.05$; TLR-2, $P = 0.05$; TLR-4, $P = 0.02$; and COX-2, $P = 0.03$) in the distal colon mucosa compared to HFD controls. However, only the raw purple-fleshed potato consuming animals had elevated GSH:GSSG ratio compared to the

HFD control (P = 0.01). Similar results were obtained in the mesenteric fat – all the potato diet consuming animals had lower expression of TLR-4 (P = 0.05), NF-κB (P = 0.05), and TNF-α (P = 0.03) compared to the HFD control. GSH:GSSG ratio was also elevated in all the purple-potato diet consuming groups and white raw group (P = 0.05) compared to the HFD control. Systemic markers of oxidative stress (8-isoprostane, P = 0.02; and 8-OHDG, P = 0.01), inflammation (TNF-α, P = 0.05; and IL-1β, P = 0.03) and serum triglycerides (P = 0.05) were suppressed in all the potato diet consuming animals compared to HFD control. However, there were no differences in the lipid profile (total cholesterol, LDL, HDL) or insulin/glucose among the HFD treatment groups. Results of this study suggest that potato consumption prevents HFD elevated oxidative stress and inflammatory biomarkers in the distal colon, mesenteric fat and the systemic circulation and there was only a marginal benefit of anthocyanin-rich potatoes over the traditional white-fleshed potatoes in this study.

5.2 Introduction

Consumption of a diet rich in fat and low in anti-oxidants is linked to elevated risk for a variety of chronic disorders. Chronic low-grade oxidative stress and inflammation are conducive for development of disorders including colon cancer and are highly affected by diet (Wisse 2004, Chuang and McIntosh 2011). Once a medical rarity in children, disorders like inflammatory bowel disease (IBD), metabolic syndrome etc. are increasingly common in kids today. Genetic and environmental factors may be responsible for driving this rise. However, as genetic makeup does not change drastically in populations over short periods of time, it could be assumed that changing environmental factors, mainly diet, are triggering new cases (Autism 2011).

Poor eating habits, mainly due to easy access to and consuming oversized portions of the high-fat, high-calorie foods, juice and soda, and inactivity, are to blame for the rise in such chronic disorders in children (Han, Lawlor et al. 2010). Despite an increasing focus on the nutritional content of school meals and multiple initiatives at state and federal government scale, children are not meeting the recommended levels of fruit and vegetable consumption (Lin and Morrison 2002). Research indicates that children (as well as adults) who eat more fruits tend to have lower risk for chronic diseases during adulthood (Lin and Morrison 2002). Both fruits and vegetables are low-fat, if not, no-fat foods when consumed straight from the orchard or garden. Instead of consuming most of their fruits raw or in juices, unadorned with sauces or fried coatings or baked in pies, higher percentage of consumption of vegetables is in the form of deep-fat frying, topped with high-fat dressings or sour cream, or including them in high-fat mixtures (Lin and Morrison 2002). The same is the case with the innocuous potato, which has been blamed for increasing risk for diabetes (Khosravi-Boroujeni, Mohammadifard et al. 2012). There is thus, a need for more research on the link between fruit and vegetable consumption and risk for chronic diseases with focus on how they are processed and consumed.

High-fat diet (HFD) elevated oxidative stress can increase the susceptibility to chronic diseases by oxidizing cellular lipids, proteins, and DNA (Matsuzawa-Nagata, Takamura et al. 2008, Fernandez-Sanchez, Madrigal-Santillan et al. 2011). Oxidative stress is involved in the link between chronic inflammation and cancer. In fact, the activation of NF-κB, a pro-inflammatory molecule and a master regulator of inflammatory gene transcription, can be blocked by antioxidants, such as cysteine, *N*-acetylcysteine, vitamin C and E, anthocyanins, thiols, and green tea polyphenols (Schulze-Osthoff, Bauer et al. 1997, Carcamo, Pedraza et al. 2002, Karlsen, Retterstol et al. 2007, Rodriguez-Ramiro, Ramos et al. 2013). NF-κB induced

secretion of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 by lymphocytes is responsible for initiating inflammation in the pathogenesis of chronic diseases such as atherosclerosis and rheumatoid arthritis (Monaco and Paleolog 2004).

The potato (*Solanum tuberosum* L.) is the fourth-most extensively grown and consumed food crop across the world after wheat, rice and maize (FAO 2009). Potato is a carbohydrate-rich, versatile vegetable prepared and served in a variety of ways world-wide. In addition to high concentrations of vitamin C and iron in the potato, the highly pigmented potato cultivars are rich in phenolic acids, anthocyanins, and carotenoids (Camire, Kubow et al. 2009). Indeed, we and others have shown that phenolic acids are present at amounts 5-12 times higher in purple-fleshed potatoes compared to their white-fleshed counterparts (Rodriguez-Saona, Giusti et al. 1998, Brown 2005, Madiwale, Reddivari et al. 2011, Radhakrishnan, Reddivari et al. 2011). However, unlike other anthocyanin-rich sources such as blueberries, potatoes are mostly processed before consumption (Brown 2005, Walton, Lentle et al. 2006, NPC 2007). It is well known that processing changes the physical and chemical composition of foods (Spanos, Wrolstad et al. 1990, Price, Bacon et al. 1997), thus, affecting their antioxidant activity (Nicoli, Anese et al. 1999, Dewanto, Wu et al. 2002). Analytical data from our lab suggested that metabolite profile of raw and baked potatoes were quite similar, however, the metabolite profile of chips was very different (Madiwale, Reddivari et al. 2012). However, it is critical to understand the effect of such processing techniques on the *in vivo* activity of the bioactive compounds in potatoes.

We hypothesized that dietary supplementation with purple-fleshed potato (raw, baked and chips) will suppress the oxidative stress/inflammation biomarkers in the distal colon, mesenteric fat and systemic circulation in a pig model. Young pigs were provided with potato supplemented diets (10 % w/w) for 13 weeks and oxidative stress and inflammatory markers

were measured. We used the pig as an animal model due to similar nutrient metabolism, and gut anatomy and physiology to humans (Cooper, Berry et al. 1997, Pond and Lei 2000). Results of the current study provide support for *in vivo* evidence for the health benefits of color-fleshed potatoes, with a focus on how it is processed.

5.3 Materials and Methods

All protocols for the use of animals in this study were approved by both the Colorado State University Institutional Animal Care and Use Committee (CSU-IACUC) and the North Carolina State University Animal Care and Use Committee. CSU-IACUC Protocol approval number 08-318A-03.

5.3.1 Animals

Sixty-four young (6 weeks old, breed: SPG) male pigs were obtained from Murphy-Brown LLC (Warsaw, NC) and were housed individually in solid concrete floor indoor pens at the North Carolina State University Swine Educational Unit (Clayton, NC). The animals were divided into 8 groups so that mean initial body weight was similar among the treatment groups (n = 8 animals/treatment). Prior to the experimental study period of 13 weeks, there was a weaning period of 3 weeks and a nursery diet period of 3 weeks. All the animals completed the study and no adverse side effects were reported.

5.3.2 Experimental Diets

Each group was given one of the following diets: Standard control diet (C1, ~5 % fat), a HFD control (C2, ~20 % added fat and ~3 % endogenous fat) and 6 different diets with the same

fat content as the HFD and 10 % of either purple or white-fleshed potato (raw, baked or chips). For the chipped diets, the fat percentage was adjusted to match the other diets. We conducted a dose response study (Chapter IV) for anti-inflammatory effects of purple-fleshed potato chips in an obese pig model and found no difference in 10 and 20 % dose in alteration of HFD elevated inflammatory markers. Thus, 10 % was the dose used in this study.

Purple-fleshed potatoes (Purple Majesty) and white-fleshed potatoes (Atlantic) were grown at Black Gold Farms (Pearsall, TX) and chips were prepared at Kettle Chips (Kettle, Salem, OR). Raw and baked potatoes were freeze dried at Vandrunen Farms, Illinois, prior to incorporation in the diet. Corn and dry fat were used as a major energy source, and soybean meal was the major protein source. Ratios between corn and soybean meal were adjusted to match energy and protein contents among diets. White corn was used to prevent carotenoids from yellow corn to affect the study. Composition of all the diets is presented in Table 5.1. Pigs consumed the experimental diets for 13 weeks; the feed and drinking water were provided ad libitum. Body weight, weight gain and feed intake were measured every week during the experimental feeding period.

Table 5.1 Composition of diets used in the study.

Ingredients (%)	Standard Control C1	High-fat Control C2	Purple Raw PR	White Raw WR	Purple Baked PB	White Baked WB	Purple Chips PC	White Chips WC
Corn	75.75	56.39	46.09	46.09	46.09	46.09	49.5	49.5
Purple-fleshed potato	0	0	10	0	10	0	10	0
White-fleshed Potato	0	0	0	10	0	10	0	10
Soybean meal w/o hulls	21	21	21	21	21	21	21	21
L-Threonine	0	0.05	0.01	0.01	0.01	0.01	0.05	0.05
L-Lysine HCl	0.15	0.2	0.15	0.15	0.15	0.15	0.18	0.18
DL-Methionine	0	0.06	0.05	0.05	0.05	0.05	0.07	0.07
Total minerals	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Vitamin mix	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Poultry fat	1	3	3	3	3	3	3	3
Dry fat	0	17.1	17.5	17.5	17.5	17.5	14	14
Dicalcium Phosphate	0.9	1	1	1	1	1	1	1
Limestone	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Composition (% unless mentioned)

Dry matter	89.6	91.7	92.6	92.6	92.6	92.6	92.2	92.2
Metabolizable energy (Cal/kg)	3388	4278	4300	4300	4300	4300	4221	4221
Crude protein	16.4	14.9	15.2	15.2	15.2	15.2	14.7	14.7
Lysine	0.84	0.84	0.84	0.84	0.84	0.84	0.83	0.83
Methionine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Tryptophan	0.16	0.15	0.16	0.16	0.16	0.16	0.15	0.15
Threonine	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Fat	4.7	23	23	23	23	23	23	23
Calcium	0.6	0.62	0.62	0.62	0.62	0.62	0.62	0.62
Phosphorus available	0.23	0.24	0.23	0.23	0.23	0.23	0.24	0.24
Phosphorus total	0.52	0.48	0.47	0.47	0.47	0.47	0.48	0.48

C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.3.3 Blood, Urine and Tissue Collection

The animals were fasted for 4-6 hours prior to sacrifice. The animals were euthanized using captive bolt method. At termination, jugular vein blood was collected without anticoagulant and serum was stored at −80 °C for biochemical analysis. Insulin, glucose and lipid analysis were performed on serum samples collected at week 11 where the animals were fasted for 12 hours. Urine was collected at the end of the study in 15 ml falcon tubes. Once collected, urine samples were snap frozen and transferred to −80 °C for long term storage prior to analysis. Two cm of the distal colon was resected and cleaned with RNAse-free phosphate-buffered saline (PBS). The mucosa was scraped using a glass slide into an RNAse free tube. The mucosa and the mesenteric fat were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

5.3.4 Tissue Oxidative Stress

Glutathione levels (GSH: reduced and GSSG: oxidized) were measured in the distal colon mucosa and mesenteric fat using UPLC/PDA (Waters ACQUITY UPLC H-Class) according to the HPLC method of Yilmaz et al (Yilmaz, Keser et al. 2009) with minor modifications.

5.3.5 Real Time Polymerase Chain Reaction

Real-time PCR analysis was used to measure the gene expression of pro-inflammatory and anti-inflammatory markers in the distal colon mucosa and the mesenteric fat. Total RNA was extracted using the Phenol-Free Total RNA Purification Kit (Amresco, Solon, OH) and cDNA synthesis was carried out using the qScript™ cDNA SuperMix (Quanta Biosciences,

Gaithersburg, MD), according to the manufacturer's instructions. Real-time PCR was performed using qPCR SYBR green supermix (Quanta Biosciences) on an Illumina Eco instrument (San Diego, CA) and mRNA expression of genes were calculated using the Eco software (Illumina). Primers used for the study are presented in Table 5.2.

Table 5.2 Primers used in the manuscript for real time PCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Product Length (bp)
Actin	GGCCGGGACCTGACCGACTA	GGAGGAGGAGGAGGCGGCC	162
Adiponectin	GCGAATGGGCATGTTAGGGA	AAATCCGGGGCAGAAAAGGA	107
COX-2	CATTGATGCCATGGAGCTGTA	CTCCCCAAAGATGGCATCTG	70
IL-10	TGATGGGGAGGATATCAAGG	TGATGGGGAGGATATCAAGG	150
NF-κB	CCCATGTAGACAGCACCACCTATGAT	ACAGAGGCTCAAAGTTCTCCACCA	132
TGF-β	CGAGCCCTGGATACCAACTA	AGGCTCCAGATGTAGGGACA	164
TLR-2	CAGTCCGGAGGTTGCATATT	ATGCTGTGAAAGGGAACAGG	137
TLR-4	ATGGCCTTTCTCTCCTGCCTGA	AGGTCCAGTATCTTGACTGATGTGGG	139
TNF-α	ATGGATGGGTGGATGAGAAA	TGGAAACTGTTGGGGAGAAG	151

5.3.6 Systemic Oxidative Stress Markers

Urine samples were analyzed for 8-isoprostane and DNA adduct 8-hydroxy-2′-deoxyguanosine following manufacturer's protocol (Cayman, Ann Harbor, MI). Urinary concentrations were normalized to creatinine levels measured using a colorimetric kit (Cayman). Serum samples were analyzed for oxidative stress marker, 8-isoprostane – free and bound following the manufacturer's protocol (Cayman). Serum oxygen radical absorbance capacity (ORAC) was calculated based on the method of Huang et al. (Huang, Ou et al. 2002).

5.3.7 Serum Inflammatory Markers

Inflammatory markers, TNF-α (Swine ELISA, Life Technologies, Grand Island, NY) and lipopolysaccharide (LPS, Limulus Amebocyte Lysate Kit, Lonza, Basel Switzerland) were analyzed in the serum based on the manufacturer's protocols. IL-1β was measured in the serum using Milliplex Luminex based multiplex immnoassay method (Millipore, Billerica, MD).

Insulin was measured using a radioimmunoassay following the manufacturer's protocol (Millipore). Glucose was measured using a commercially available kit (Cayman) following standard recommended protocol. Both insulin and glucose were measured after a 12 hour fast at week 11 of the study. Serum lipid profile (Total Cholesterol, HDL, LDL and Triglycerides) were also measured at week 11 using standard protocols on a Beckman - Olympus AU400e Chemistry Analyzer at the University of Colorado Hospital.

5.3.8 Statistical Analysis

Completely randomized block design was used in this study. Individual pigs were the experimental unit. Data were analyzed using the MIXED procedure in SAS (SAS Institute, Cary,

NC). $P \leq 0.05$ was considered significant. The results were expressed as means ± S.E. for each treatment group.

5.4 Results

5.4.1 Physical Parameters

Initial body weight of pigs did not differ among treatments. There were no significant differences in the overall feed intake/day and weight gain/day among the HFD treatment groups at the end of the study (Table 5.3). It should be noted that the overall feed conversion ratio, feed consumed/weight gain, did not differ among the all treatment groups at the end of the study (Appendix IV). There were no differences in the weight at the end of the study among the HFD treatment groups (excluding PB, $P \leq 0.05$). No significant differences were observed among treatment groups in back-fat thickness (Appendix V). All the animals completed the study, and no adverse side effects/infections were reported.

Table 5.3 Body weight (in kg, top panel), average daily gain (in kg/day, middle panel) and average daily feed intake (in kg/day, bottom panel) of animals in the prevention study. Data are presented as means ± S.E of 8 animals per group.

Dietary Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13
Body Weight (kg)														
C1	22.7 ± 1.3[a]	26.8 ± 1.7[a]	38.7 ± 2.4[a]	48.7 ± 2.9[a]	62.2 ± 3.5[a]	74.3 ± 4.3[a]	91.7 ± 4.5[a]	105.8 ± 5.1[a]	125.3 ± 5.4[a]	140.9 ± 5.8[a]	162.9 ± 6[a]	181.4 ± 6.2[a]	198 ± 5.2[a]	216.2 ± 7.3[a]
C2	22.8 ± 1[a]	27.3 ± 1[a]	35.2 ± 1.2[ab]	43 ± 1.8[b]	54.2 ± 2.4[b]	64 ± 3.3[b]	78.2 ± 3.5[b]	89.2 ± 3.9[b]	105.6 ± 4.8[c]	117.7 ± 5.3[b]	136.4 ± 6.5[b]	154.2 ± 6.6[b]	169.1 ± 7.1[b]	185.8 ± 7.8[b]
PR	22.6 ± 1[a]	26.9 ± 1.3[a]	35.3 ± 1.2[ab]	42.8 ± 1.4[b]	55.8 ± 1.8[b]	66 ± 2.3[b]	81.3 ± 3.1[b]	92.2 ± 4.1[b]	109.2 ± 5.5[bc]	121.3 ± 6.9[b]	137.9 ± 8.8[b]	154.3 ± 10.1[b]	169.6 ± 11[b]	181.6 ± 13.1[b]
WR	22.7 ± 1[a]	27.3 ± 1[a]	36.6 ± 1.1[ab]	45.1 ± 1.5[ab]	57.1 ± 1.9[b]	67.8 ± 2.3[b]	81.6 ± 2.3[b]	92.3 ± 2.7[b]	108.8 ± 2.9[bc]	121.8 ± 3.3[b]	138.2 ± 4.2[b]	153 ± 4.3[b]	167.8 ± 5.2[b]	182.1 ± 6.5[b]
PB	22.8 ± 0.9[a]	27 ± 0.9[a]	35.9 ± 1.5[ab]	44 ± 1.9[b]	58.4 ± 2.2[ab]	71 ± 3.5[ab]	87.2 ± 3.1[a]	99.3 ± 3.7[ab]	120.3 ± 4.2[a]	135.9 ± 5.3[a]	155.9 ± 5.7[a]	175.4 ± 5.8[a]	191.1 ± 5.6[a]	208.9 ± 6.9[a]
WB	22.6 ± 1[a]	27.3 ± 1.1[a]	35.4 ± 1.9[ab]	43.6 ± 2.7[b]	55.7 ± 3.2[b]	65.8 ± 4.3[b]	81 ± 4.4[b]	92.4 ± 4.9[b]	110.1 ± 5.7[b]	124.2 ± 6.6[ab]	142.8 ± 7.8[b]	159 ± 7.6[b]	175.6 ± 8.6[b]	192.6 ± 10.3[b]
PC	22.5 ± 0.7[a]	27.3 ± 0.8[a]	35.7 ± 0.8[ab]	43.1 ± 0.9[b]	56 ± 1[b]	67.3 ± 1.8[b]	82.9 ± 1.9[b]	94.4 ± 2.9[b]	112.2 ± 3.6[bc]	127.8 ± 4.3[ab]	147.8 ± 4.4[ab]	167.2 ± 5.6[ab]	183 ± 6.1[ab]	197.1 ± 7.8[ab]
WC	22.6 ± 0.8[a]	25.8 ± 1[a]	34.7 ± 0.9[b]	43.5 ± 1.1[b]	56.8 ± 1.3[b]	68.6 ± 1.8[ab]	85.9 ± 2.7[ab]	99.5 ± 2.9[ab]	118.5 ± 3.7[ab]	130.7 ± 4.5[ab]	150.2 ± 6.1[ab]	167.8 ± 6.5[ab]	184.8 ± 6.4[ab]	199.4 ± 7.7[ab]

	Average Daily Gain (kg/d)													Overall Daily Gain (kg/d)
C1	0.6 ± 0.1 ^{ab}	1.5 ± 0.1 ^{a}	1.4 ± 0.1 ^{a}	1.7 ± 0.1 ^{a}	1.5 ± 0.1 ^{a}	2.9 ± 0.1 ^{a}	2 ± 0.1 ^{a}	2.4 ± 0.1 ^{a}	2.2 ± 0.1 ^{a}	3.1 ± 0.2 ^{a}	2.3 ± 0.2 ^{a}	2.1 ± 0.2 ^{a}	2.6 ± 0.4 ^{a}	2.2 ± 0.1 ^{a}
C2	0.6 ± 0 ^{ab}	1 ± 0 ^{b}	1.1 ± 0.1 ^{b}	1.4 ± 0.1 ^{b}	1.2 ± 0.1 ^{b}	2.4 ± 0.2 ^{b}	1.6 ± 0.2 ^{b}	2 ± 0.1 ^{b}	1.7 ± 0.1 ^{b}	2.7 ± 0.2 ^{ab}	2.2 ± 0.1 ^{a}	1.9 ± 0.1 ^{a}	2.4 ± 0.2 ^{a}	1.8 ± 0.1 ^{b}
PR	0.6 ± 0.1 ^{ab}	1.1 ± 0.1 ^{b}	1.1 ± 0.1 ^{b}	1.6 ± 0.1 ^{ab}	1.3 ± 0.1 ^{ab}	2.6 ± 0.2 ^{ab}	1.6 ± 0.2 ^{b}	2.1 ± 0.2 ^{ab}	1.7 ± 0.2 ^{b}	2.4 ± 0.3 ^{b}	2 ± 0.2 ^{a}	1.9 ± 0.2 ^{a}	1.7 ± 0.3 ^{b}	1.8 ± 0.1 ^{b}
WR	0.6 ± 0 ^{ab}	1.2 ± 0.1 ^{b}	1.2 ± 0.1 ^{ab}	1.5 ± 0.1 ^{ab}	1.3 ± 0.1 ^{ab}	2.3 ± 0.2 ^{b}	1.5 ± 0.1 ^{b}	2.1 ± 0.1 ^{ab}	1.9 ± 0.1 ^{ab}	2.3 ± 0.2 ^{b}	1.9 ± 0.2 ^{a}	1.8 ± 0.1 ^{a}	2 ± 0.2 ^{ab}	1.8 ± 0.1 ^{b}
PB	0.6 ± 0 ^{ab}	1.1 ± 0.1 ^{b}	1.2 ± 0.1 ^{ab}	1.8 ± 0.1 ^{b}	1.6 ± 0.2 ^{a}	2.7 ± 0.1 ^{ab}	1.7 ± 0.1 ^{b}	2.6 ± 0.1 ^{a}	2.2 ± 0.2 ^{b}	2.9 ± 0.2 ^{a}	2.4 ± 0.2 ^{a}	2 ± 0.2 ^{a}	2.5 ± 0.2 ^{a}	2.1 ± 0.1 ^{ab}
WB	0.7 ± 0 ^{b}	1 ± 0.1 ^{b}	1.2 ± 0.1 ^{ab}	1.5 ± 0.1 ^{ab}	1.3 ± 0.2 ^{ab}	2.5 ± 0.1 ^{b}	1.6 ± 0.1 ^{b}	2.2 ± 0.1 ^{ab}	2 ± 0.2 ^{ab}	2.7 ± 0.2 ^{ab}	2 ± 0.1 ^{a}	2.1 ± 0.2 ^{a}	2.4 ± 0.3 ^{a}	1.9 ± 0.1 ^{b}
PC	0.7 ± 0 ^{b}	1.1 ± 0.1 ^{b}	1.1 ± 0.1 ^{b}	1.6 ± 0.1 ^{ab}	1.4 ± 0.1 ^{ab}	2.6 ± 0.1 ^{ab}	1.6 ± 0.2 ^{b}	2.2 ± 0.2 ^{ab}	2.2 ± 0.2 ^{ab}	2.9 ± 0.2 ^{b}	2.4 ± 0.2 ^{a}	2 ± 0.1 ^{a}	2 ± 0.3 ^{ab}	1.9 ± 0.1 ^{b}
WC	0.4 ± 0.2 ^{a}	1.1 ± 0.1 ^{b}	1.3 ± 0.1 ^{ab}	1.7 ± 0.1 ^{ab}	1.5 ± 0.1 ^{ab}	2.9 ± 0.2 ^{a}	1.9 ± 0.1 ^{ab}	2.4 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	2.8 ± 0.3 ^{ab}	2.2 ± 0.2 ^{a}	2.1 ± 0.2 ^{a}	2.1 ± 0.2 ^{ab}	1.8 ± 0.1 ^{b}

	Average Daily Feed Intake (kg/d)												Overall Daily Feed Intake (kg/d)
C1	1.9 ± 0.1 ^{a}	4.2 ± 0.7 ^{ab}	2.3 ± 0.2 ^{a}	3.5 ± 0.2 ^{a}	4.5 ± 0.2 ^{a}	5 ± 0.2 ^{a}	5.2 ± 0.2 ^{a}	4.9 ± 0.2 ^{a}	6.2 ± 0.2 ^{a}	6.3 ± 0.2 ^{a}	4.5 ± 0.1 ^{a}	8.5 ± 0.4 ^{a}	4.8 ± 0.2 ^{a}
C2	1.4 ± 0.1 ^{b}	4.5 ± 0.3 ^{ab}	1.9 ± 0.1 ^{a}	2.6 ± 0.1 ^{b}	3.4 ± 0.2 ^{b}	3.6 ± 0.2 ^{b}	3.8 ± 0.1 ^{b}	3.6 ± 0.2 ^{b}	4.4 ± 0.3 ^{b}	4.8 ± 0.3 ^{b}	3.8 ± 0.2 ^{b}	6.5 ± 0.2 ^{b}	3.7 ± 0.2 ^{b}
PR	1.3 ± 0.1 ^{a}	4.6 ± 0.3 ^{ab}	1.9 ± 0.1 ^{a}	2.9 ± 0.2 ^{bc}	3.6 ± 0.2 ^{b}	3.8 ± 0.2 ^{b}	4.1 ± 0.3 ^{b}	3.7 ± 0.4 ^{b}	4.4 ± 0.4 ^{b}	4.7 ± 0.4 ^{b}	3.7 ± 0.3 ^{b}	5.5 ± 0.5 ^{c}	3.7 ± 0.3 ^{b}
WR	1.6 ± 0.1 ^{ab}	4.6 ± 0.2 ^{ab}	2.1 ± 0.1 ^{a}	3 ± 0.1 ^{c}	3.7 ± 0.1 ^{b}	3.9 ± 0.1 ^{b}	4.1 ± 0.1 ^{b}	4.1 ± 0.2 ^{b}	4.5 ± 0.2 ^{b}	4.7 ± 0.2 ^{b}	4 ± 0.2 ^{b}	6 ± 0.3 ^{bc}	3.9 ± 0.1 ^{b}
PB	1.4 ± 0.1 ^{a}	4.9 ± 0.3 ^{a}	2.2 ± 0.1 ^{a}	3.2 ± 0.2 ^{ac}	3.9 ± 0.1 ^{b}	4.1 ± 0.1 ^{b}	4.8 ± 0.1 ^{a}	4.5 ± 0.2 ^{a}	5.3 ± 0.3 ^{c}	5.6 ± 0.2 ^{c}	4.2 ± 0.2 ^{ab}	6.5 ± 0.5 ^{bc}	4.2 ± 0.2 ^{b}
WB	1.4 ± 0.1 ^{a}	4.5 ± 0.3 ^{ab}	1.9 ± 0.1 ^{a}	2.8 ± 0.2 ^{bc}	3.7 ± 0.2 ^{b}	3.8 ± 0.2 ^{b}	4.2 ± 0.2 ^{b}	4.2 ± 0.2 ^{ab}	4.8 ± 0.3 ^{bc}	4.9 ± 0.2 ^{b}	4.1 ± 0.3 ^{ab}	6.7 ± 0.5 ^{b}	3.9 ± 0.2 ^{b}
PC	1.4 ± 0.1 ^{a}	4.5 ± 0.3 ^{ab}	1.9 ± 0.1 ^{a}	2.8 ± 0.1 ^{bc}	3.4 ± 0.2 ^{b}	3.6 ± 0.2 ^{b}	3.9 ± 0.2 ^{b}	3.9 ± 0.2 ^{b}	4.6 ± 0.2 ^{b}	4.9 ± 0.2 ^{b}	3.9 ± 0.2 ^{b}	5.6 ± 0.3 ^{c}	3.7 ± 0.1 ^{b}
WC	1.4 ± 0.1 ^{a}	4.2 ± 0.2 ^{b}	1.9 ± 0.1 ^{a}	2.8 ± 0.2 ^{bc}	3.7 ± 0.2 ^{b}	4 ± 0.1 ^{b}	4.2 ± 0.2 ^{b}	3.5 ± 0.2 ^{b}	4.3 ± 0.3 ^{b}	4.7 ± 0.2 ^{b}	3.9 ± 0.2 ^{b}	5.6 ± 0.4 ^{c}	3.9 ± 0.1 ^{b}

Results were expressed as mean ± SE for 7-8 animals in each treatment group. Means in columns (within a week between treatment groups) that differ by a common letter (a, b, c) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively, at 10 % supplementation.

5.4.2 Colonic GSH:GSSG Ratio

Glutathione is an intracellular anti-oxidant in cells. Glutathione exists in two forms reduced (GSH) and oxidized (GSSG). Almost 90 % of glutathione exists in the reduced form, however, in conditions of oxidized stress, GSSG content of the cells increases. Therefore, a suppressed ratio of GSH:GSSG is an indicator of elevated oxidative stress (Owen and Butterfield 2010). We measured GSH:GSSG ratio in the distal colon of the animals. All the potato groups irrespective of color/processing had levels similar to the standard diet animals. Only the PR group had significantly higher GSH:GSSG ratio compared to the HFD control (P = 0.01, Figure 5.1).

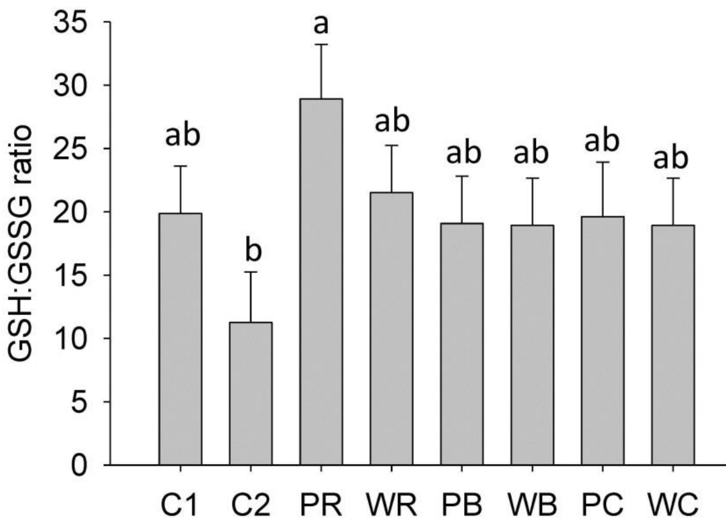

Figure 5.1. Reduced (GSH) and oxidized (GSSG) glutathione were measured in the distal colon mucosa using UPLC/PDA. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.4.3 Colonic Inflammatory Markers

We measured relative expression of innate inflammatory markers TLRs 2, 4 and NF-κB in the distal colon mucosa of the animals using quantitative real-time PCR. Expression of TLRs 2, 4 and NF-κB were elevated in the HFD group compared to standard animals. All the animals consuming potato diets had suppressed TLRs 2, 4 and NF-κB expression compared to levels in HFD animals, to levels similar to the standard group ($P = 0.05$, $P = 0.02$, and $P = 0.04$, respectively, Figure 5.2A). Similar results were obtained for TNF-α and COX-2 ($P = 0.05$ and $P = 0.03$, respectively, Figures 5.2B). Combined with the results obtained in GSH:GSSG ratio, we show that bioactive components (e.g. phenolic acids, vitamin C, resistant starch etc.) in the white-fleshed potatoes were sufficient to prevent HFD induced oxidative stress/inflammation in the distal colon. In addition, experimental diets did not alter the relative expression of anti-inflammatory cytokines IL-10 and TGF-β in the distal colon mucosa of these animals (Figure 5.3).

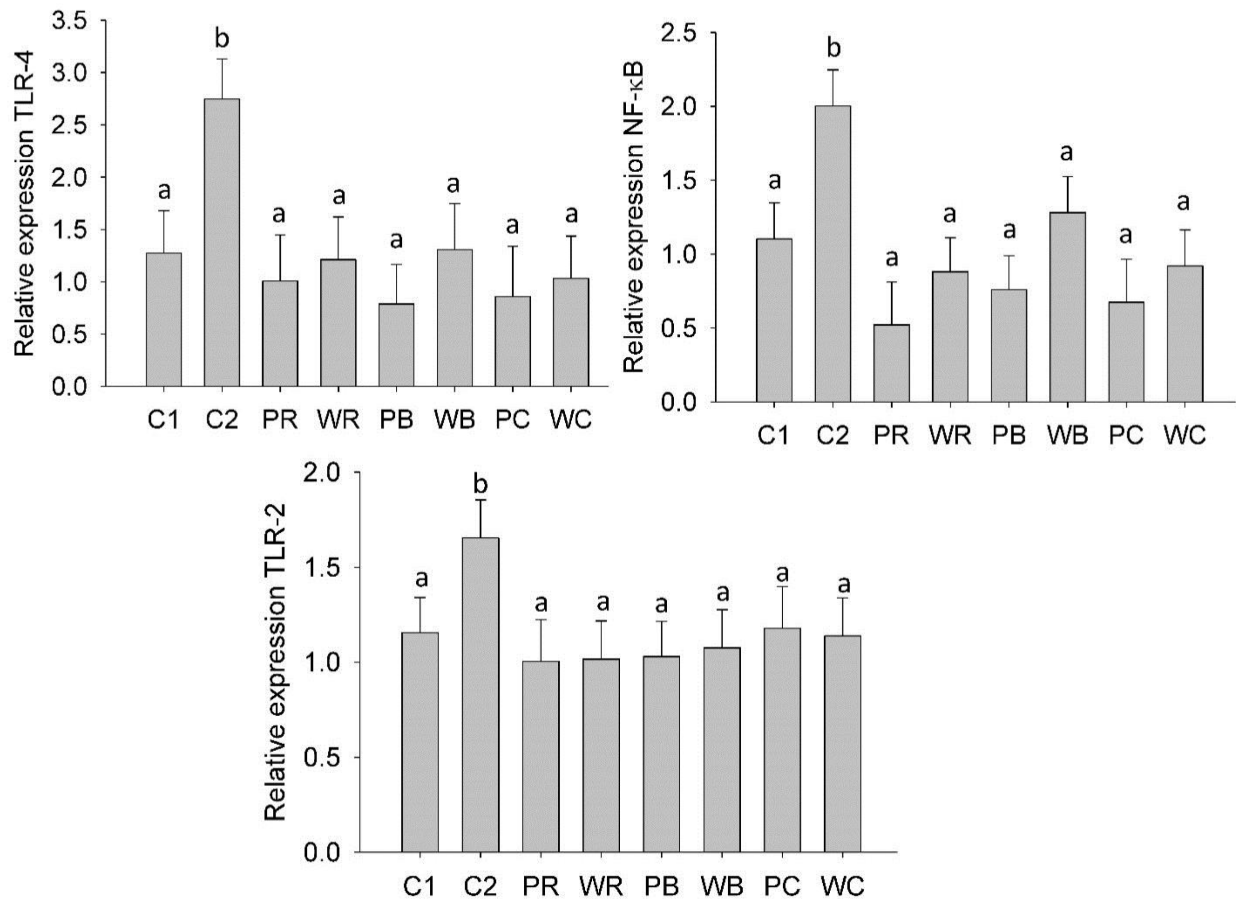

Figure 5.2A Relative expression of TLR-4, NF-κB and TLR-2 as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

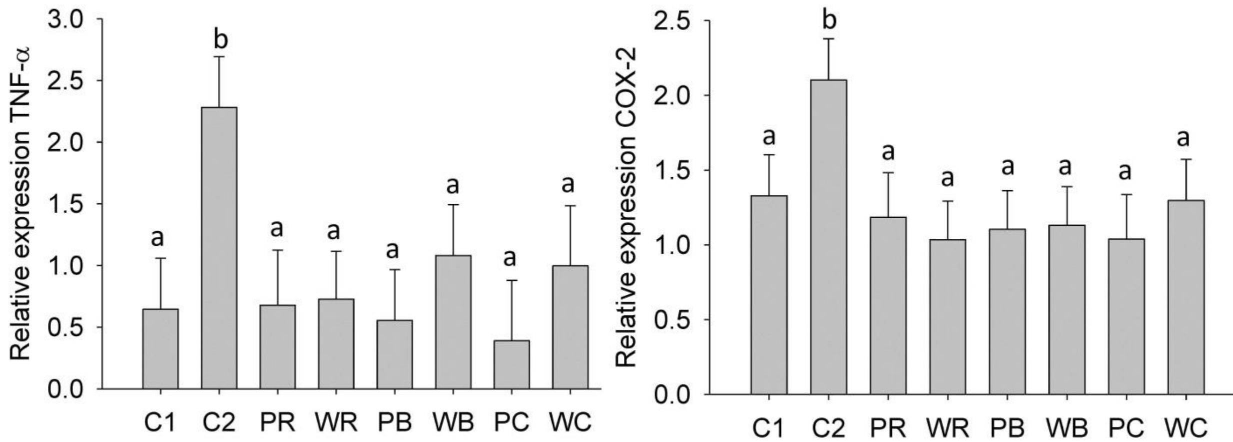

Figure 5.2B Relative expression of TNF-α and COX-2 as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

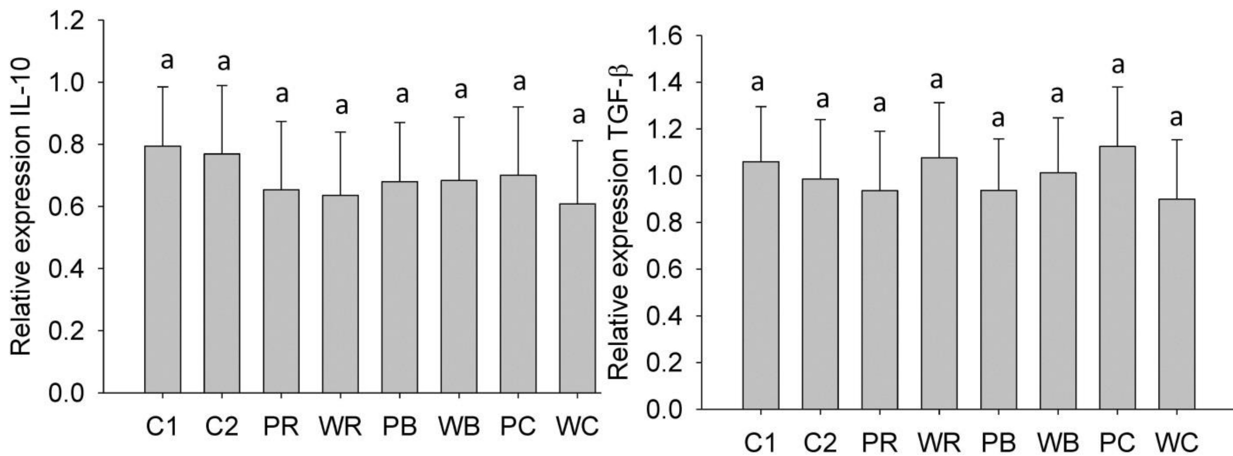

Figure 5.3 Relative expression of IL-10 and TGF-β as measured using real time PCR in the distal colon mucosa. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2,

high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.4.4 Mesenteric Fat GSH:GSSG Ratio

In the mesenteric fat, there were no significant differences in the GSH:GSSG ratio, a marker of oxidative stress, between the standard control group and the HFD group. All the animals consuming purple-fleshed potato (PR, PB and PC) and the animals consuming white-fleshed potato (WR) had significantly elevated GSH:GSSG ratio compared to HFD control suggesting potent anti-oxidant efficacy of purple-fleshed potatoes in the distal colon even after processing (P = 0.05). GSH:GSSG ratio in the processed white-fleshed potato groups (WB, WC) was not significantly different from HFD control (Figure 5.4).

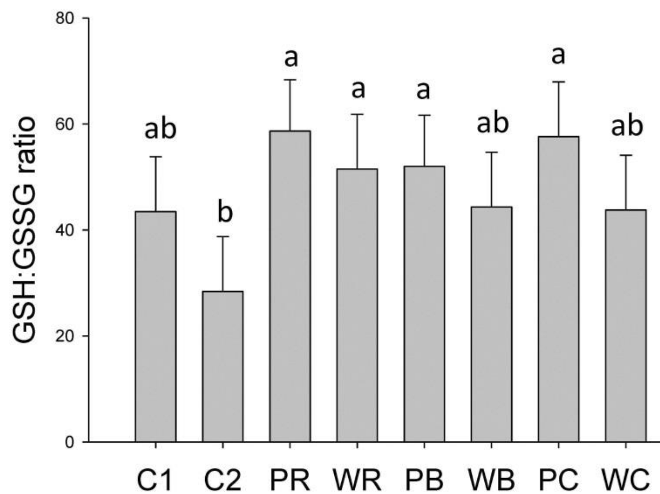

Figure 5.4 Glutathione (GSH-reduced and GSSG-oxidized) were measured in the mesenteric fat using UPLC/PDA. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.4.5 Mesenteric Fat Inflammatory Markers

We measured relative expression of the innate inflammatory markers in the mesenteric fat. Animals consuming HFD had elevated TLR-4 and NF-κB expression compared to standard animals. All the animals on potato diets had significantly suppressed TLR-4 and NF-κB expression compared to animals on HFD (Figure 5.5A) suggesting an anti-inflammatory role of potato, even after processing. Expression of pro-inflammatory cytokine TNF-α was elevated in the HFD group compared to standard animals. All the animals on the potato diets had suppressed TNF-α expression compared to levels in HFD animals ($P = 0.03$, Figure 5.5B). Mesenteric fat expression of adiponectin, an anti-inflammatory adipokine, was not significantly altered between groups (Figure 5.5B). In addition, we measured mesenteric fat relative expression of anti-inflammatory cytokines IL-10 and TGF-β. We found no significant differences among the groups (Figure 5.6).

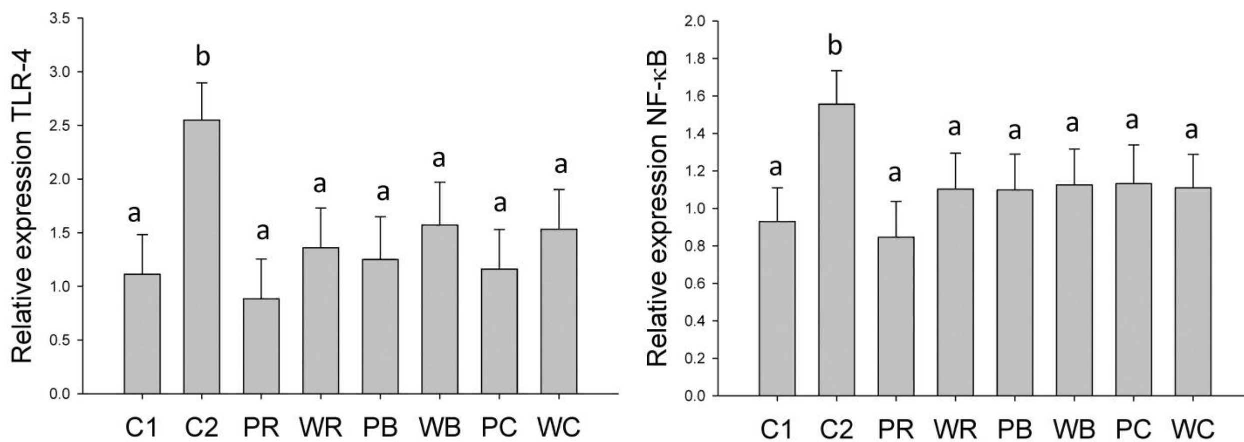

Figure 5.5A Relative expression of TLR-4 and NF-κB as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat

diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

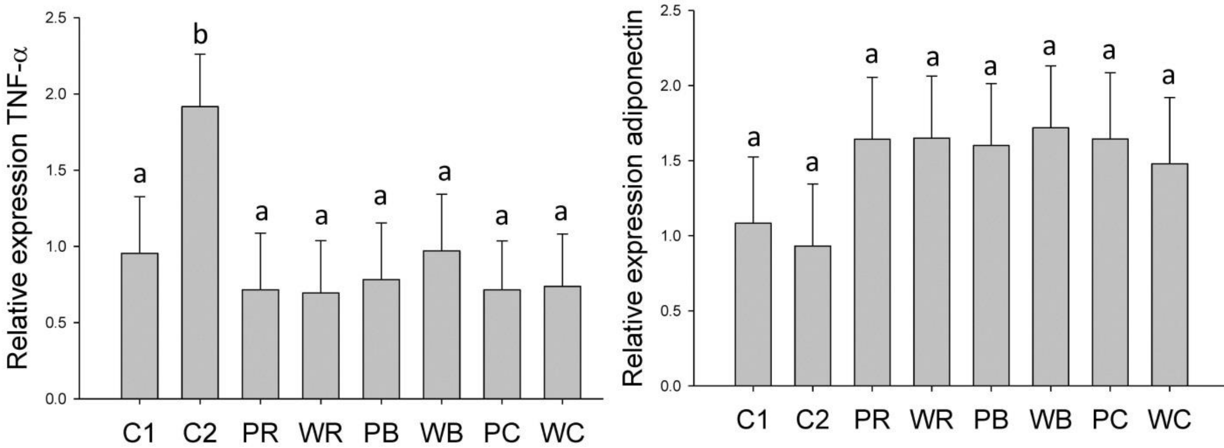

Figure 5.5B Relative expression of TNF-α and adiponectin as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, at 10 % supplementation.

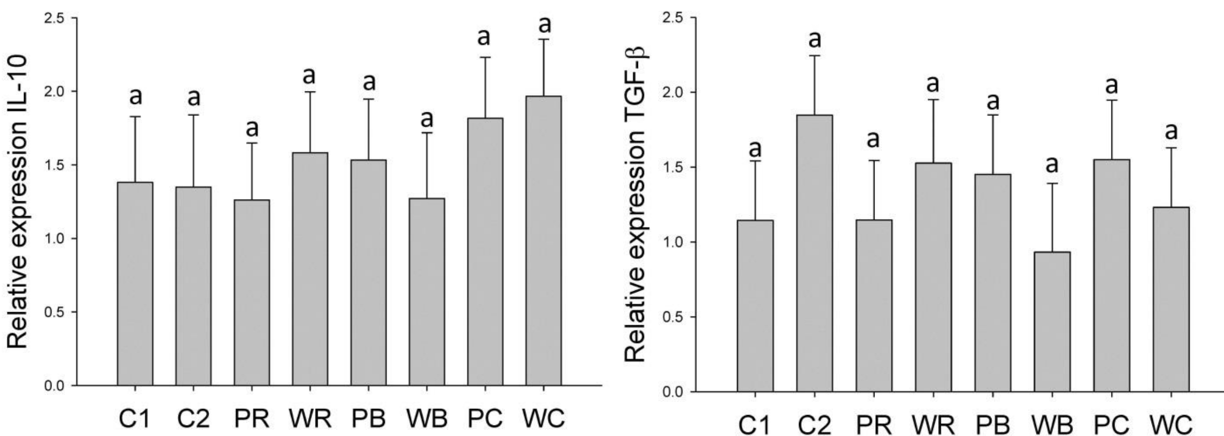

Figure 5.6 Relative expression of IL-10 and TGF-β as measured using real time PCR in the mesenteric fat. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group.

Means that differ by a common letter differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.4.6 Systemic Oxidative Stress

Systemic oxidative stress biomarkers were measured in serum/urine at the end of the study. Urinary 8-isoprostane concentrations, normalized to creatinine levels, were elevated in the HFD group compared to standard animals (Figure 5.7A). All the potato diets consuming animals had lower urinary 8-isoprostane concentrations, suggesting potato diets suppressed systemic oxidative stress ($P = 0.02$). Similar to 8-isoprostane, urinary 8-OHDG, DNA adduct, was also elevated in the HFD control group compared to standard control; all the potato diet consuming animals had lower urinary 8-OHDG compared to the HFD group ($P = 0.01$, Figure 5.7A). In addition, the levels of urinary 8-isoprostane and 8-OHDG in the potato groups were similar to the standard diet group animals (Figure 5.7A). We measured serum concentrations of 8-isoprostane (free and bound), however, there were no significant differences among the treatment groups (Appendix VI). Potato consumption did not significantly elevate serum total anti-oxidant capacity (Total ORAC, Appendix VI).

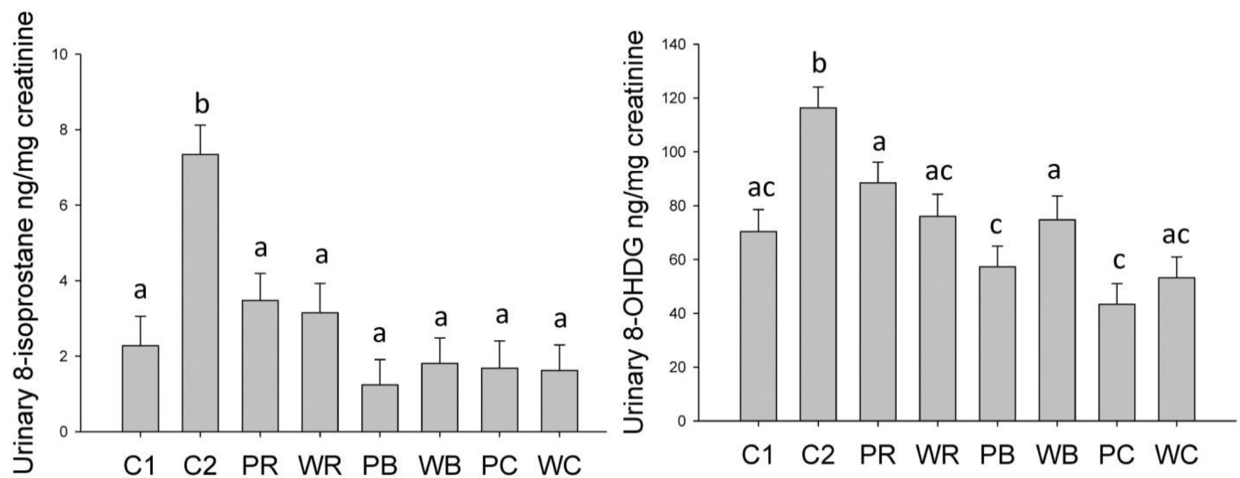

Figure 5.7A 8-isoprostane and 8-OHDG concentrations in porcine urine were measured using ELISA. Results were normalized to levels of creatinine. ELISA was performed according to manufacturer's protocol (Cayman). Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b, c) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets and R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

5.4.7 Systemic Inflammatory Markers

Systemic inflammation biomarkers were measured at the end of the study. Serum TNF-α concentration was significantly elevated in the HFD animals compared to standard control animals (Figure 5.7B). Similar to the oxidative stress markers, all the potato diets significantly suppressed serum TNF-α levels compared to the HFD controls ($P = 0.05$, Figure 5.7B). We also measured IL-1β, IL-4, IL-6, IL-8 and IL-10 in the serum of these animals using a porcine milliplex multiplex immunoassay. Trends in the level of IL-1β in the serum of pigs mirrored TNF-α level. HFD consuming animals had elevated IL-1β compared to standard control and all

the animals on potato diets had suppressed serum IL-1β compared to HFD group (P = 0.03, Figure 5.7B). There were no significant differences between groups in serum concentrations of IL-4, IL-6, IL8 and IL-10 (data not shown). We measured insulin and glucose in the serum of these animals from week 11 of the study. There were no significant differences (Figure 5.7B) among the groups in serum insulin and glucose (P ≤ 0.05).

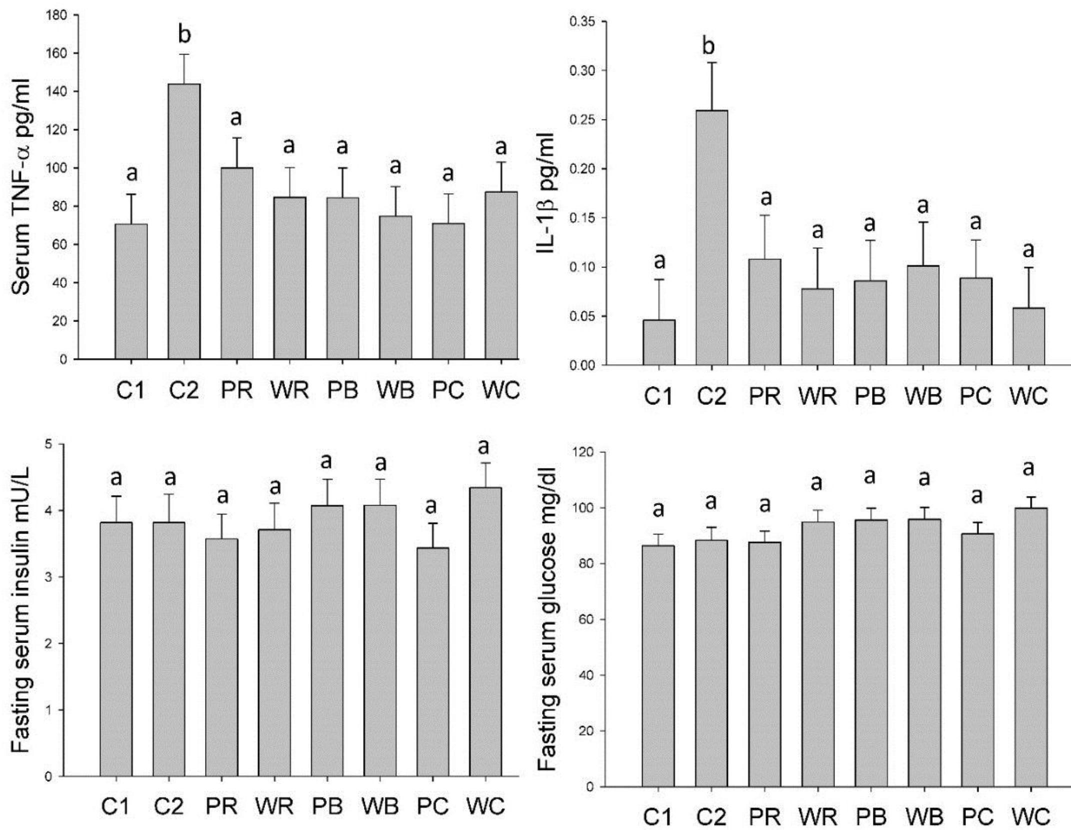

Figure 5.7B TNF-α was measured in porcine serum using ELISA following manufacturers protocol (Life Technologies). IL-1β cytokine levels were measured in porcine serum using Millipore Milliplex kit following manufacturers protocol (Millipore). Insulin was measured using a radioimmunoassay following the manufacturers protocol (Millipore). Glucose was measured using a commercially available kit (Cayman). Results were expressed as mean ± SE for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ (P ≤

0.05). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation.

Serum lipids were measured to assess whether the potato diets improved the lipid profile of these animals. This profile was measured at week 11 of the study. There were no significant differences in the total cholesterol, HDL and LDL among the HFD consuming treatment groups. However, total cholesterol and HDL levels were significantly higher in the HFD animals compared to the levels in the standard diet animals (Figure 5.7C, $P \leq 0.05$). The serum triglyceride levels followed similar trend of the inflammatory markers. The HFD control animals had elevated serum triglycerides compared to the standard control and all the potato diets consuming animals had significantly lower serum triglyceride levels compared to HFD control and were not different from the standard animals ($P = 0.05$, Figure 5.7C).

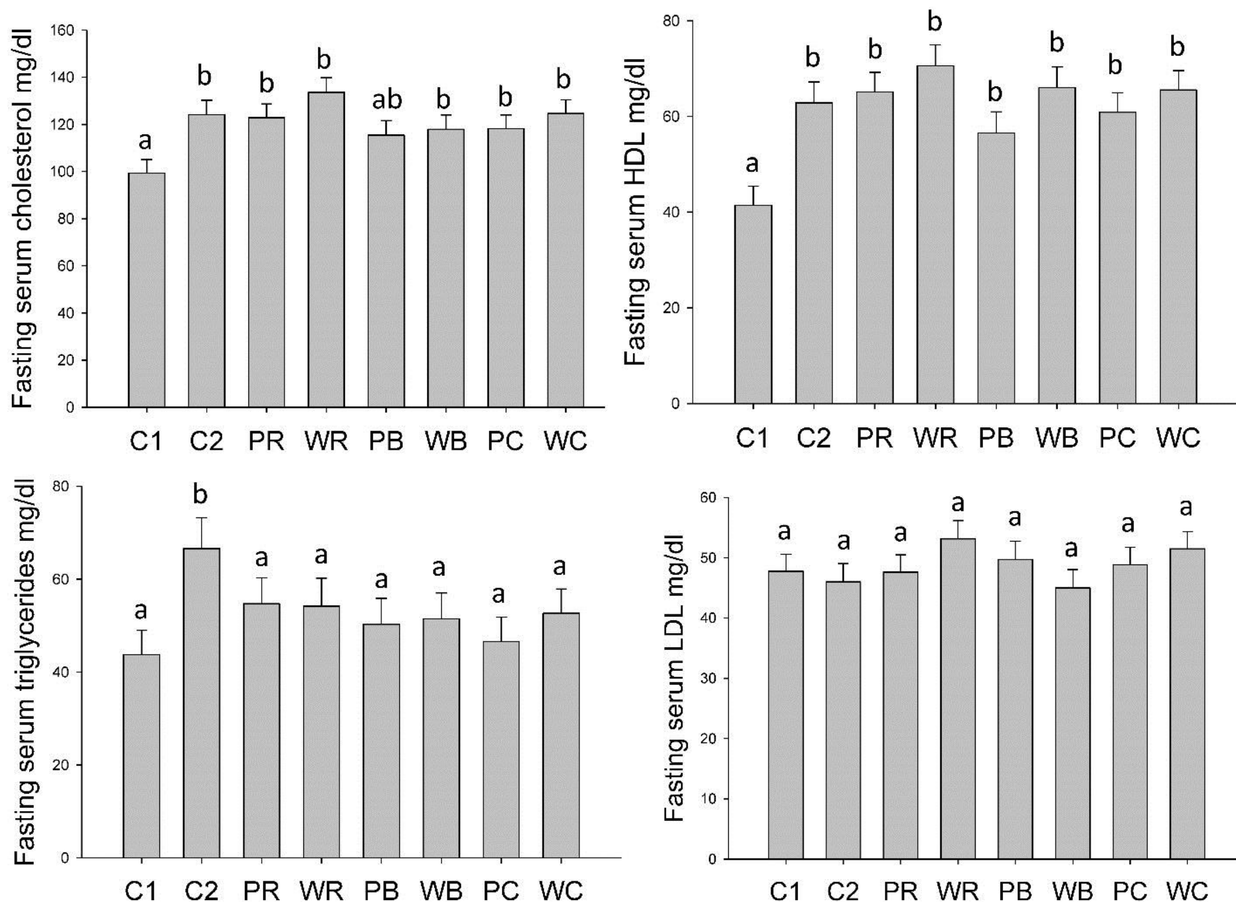

Figure 5.7C Serum lipids profile (Total cholesterol, HDL-direct, LDL and triglycerides) were measured on a Beckman-Olympus AU400e Chemistry Analyzer at the University of Colorado Hospital facility. Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. Means that differ by a common letter (a, b) differ ($P \leq 0.05$). C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively, at 10 % supplementation.

5.5 Discussion

Results of our earlier reversal study (Chapter IV) demonstrated that only the consumption of purple-fleshed potato suppressed oxidative stress and inflammatory markers in the distal colon, mesenteric fat and systemic circulation in HFD consuming pigs. Current results demonstrate that purple-fleshed potato supplementation in the diet of the HFD consuming young pigs prevented the increase in oxidative stress and inflammation biomarkers due to HFD consumption. White-fleshed potato consuming animals showed suppressed levels of oxidative stress and inflammation markers compared to the HFD controls. Results suggest that potato diets retain anti-oxidant and anti-inflammatory properties *in vivo* even after processing.

5.5.1 Purple-Fleshed Potato Prevented Colonic and Mesenteric Fat Oxidative Stress (GSH:GSSG Ratio) in HFD Consuming Animals.

Glutathione (GSH) is the most abundant anti-oxidant in aerobic cells, present in millimolar (mM) concentrations in tissue. GSH is critical for protecting various tissues from oxidative stress, acting as a free radical scavenger and is an inhibitor of lipid peroxidation. GSH participates in the detoxification of hydrogen peroxide by various glutathione peroxidases. When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease. Therefore, the determination of the GSH:GSSG ratio is a useful indicator of oxidative stress in animal and human tissues (Owen and Butterfield 2010).

Our results demonstrate that only the animals consuming purple-fleshed potatoes (raw, PR) had significantly higher distal colonic GSH:GSSG ratio compared to HFD controls. GSH:GSSG ratio in the other groups including the standard control, were numerically higher but not significantly different ($P \leq 0.05$) compared to the HFD controls (Figure 5.1). Results in the

mesenteric fat were similar to colon. In the mesenteric fat, all the purple-fleshed potato groups (PR, PB and PC) and the WR group had significantly elevated GSH:GSSG ratio compared to the HFD control animals ($P \leq 0.05$, Figure 5.4). These results suggest that potato supplementation resulted in the prevention of the increased oxidative stress markers in the HFD consuming animals. Although, low doses of phenolics are present in the white-fleshed potato, data suggests that it was sufficient to positively alter the glutathione redox status in the young animals. There was however, a marginal benefit of purple-fleshed potato consumption (Figure 5.1, 5.4).

5.5.2 Purple and White-Fleshed Potato Prevented HFD Elevated Colonic and Mesenteric Fat Inflammatory Markers.

HFD induced inflammation in the gut has been extensively explored in recent studies (Ding, Chi et al. 2010, Kim, Gu et al. 2012). HFD induced altered gut bacteria and TLR activation are key elements for induction of the innate inflammatory response via triggering signaling cascades including the transcription factor NF-κB (Takeda and Akira 2004). Thus, we measured expression of TLR-2 (flagellin receptor), TLR-4 (LPS receptor) and NF-κB in the colonic mucosa in these animals. Expression of TLR-2, TLR-4 and NF-κB were suppressed in the potato consuming animals compared to the HFD control. In addition, these groups had levels similar to that of the standard animals suggesting an overall benefit of potato consumption (Figure 5.2A). We measured expression of TLR-4 and NF-κB in the mesenteric fat. HFD controls had elevated expression of TLR-4 and NF-κB compared to the standard controls in the mesenteric fat. All the potato diet consuming animals, irrespective of color/processing, had significantly lower expression levels of TLR-4 and NF-κB compared to HFD controls, to levels similar to the standard controls. Results suggest an overall benefit of potato consumption (Figure 5.5A).

Pro-inflammatory markers including cytokines such as IL-1β, TNF-α and IL-6 are increased in colitis in the colonic mucosa (Berg and Scherer 2005). Studies have suggested that adipokines secreted by adipose tissue (TNF-α, IL-6, adiponectin etc.) are closely associated with chronic inflammatory disorders such as ulcerative colitis (Li, Lelliott et al. 2008, Teixeira, Leonel et al. 2011). Thus, during inflammatory conditions of the colon such as colitis; both the colonic and the mesenteric fat show a pro-inflammatory profile (Li, Lelliott et al. 2008, Teixeira, Leonel et al. 2011) indexed by over-production of TNF-α (Zhang, Xu et al. 2006, Gambero, Marostica et al. 2007, Kim, Gu et al. 2012). In our study, results indicate that the potato consumption suppressed expression of TNF-α in both the distal colonic mucosa and the mesenteric fat compared to the HFD controls (Figure 5.2B, 5.5B). Levels in the potato groups were similar to the standard control. Mesenteric fat expression of adiponectin was not different among groups (Figure 5.5B).

High dietary intakes of total fats, omega-6 fatty acids, and meat were shown to be associated with an increased risk of CD and UC, colonic inflammatory disorders. High fiber and fruit intakes were associated with decreased CD risk, and high vegetable intake was associated with decreased UC risk (Hou, Abraham et al. 2011). Bioactive compounds in potatoes such as anthocyanins and phenolic acids have shown anti-oxidant and anti-inflammatory properties in many models (Luceri, Guglielmi et al. 2004, Piberger, Oehme et al. 2011, Wu, Xu et al. 2011). Similarly, our results support an anti-inflammatory role of potato consumption.

Since under homeostatic conditions a balance is maintained between pro- and anti-inflammatory indices, we measured relative expression of anti-inflammatory cytokines IL-10 and TGF-β in the distal colon mucosa and the mesenteric fat of these animals. There were no significant differences among groups in the expression of anti-inflammatory cytokines IL-10 and

TGF- β (Figure 5.3, 5.6) suggesting that potatoes exhibit anti-inflammatory action via targeting pro-inflammatory molecules. We measured expression of TLR-4, NF-κB and TNF-α in the proximal colon and ileum mucosa of these animals. We did not detect any significant difference among the groups (Appendix VII). There were no differences even in the expression of TLR-4, NF-κB and TNF-α between the standard and the HFD controls in the proximal colon and the ileum mucosa. This might suggest that HFD targets the distal colon in the pig model. Although, some studies support this observation (Lam, Mitchell et al. 2011), we are currently using advanced metabolomics and pyrosquencing (gut bacteria) methods to confirm this observation.

5.5.3 Potato Diets Suppressed Systemic Oxidative Stress Markers in HFD Consuming Animals.

8-Isoprostane is a prostaglandin-F2-like compound that belongs to the F2 isoprostane class. It is produced *in vivo* by the free radical-catalyzed peroxidation of arachidonic acid. Concentrations of 8-isoprostane, one of the more prevalent F_2 isoprostanes, in the plasma/serum and urine are regarded as one of the best indices of lipid peroxidation, and oxidative stress that are currently available (Montuschi, Barnes et al. 2004). In our study, we measured 8-isoprostane in the serum, and in the urine of the HFD consuming pigs. All potato diets suppressed urinary 8-isoprostane concentrations compared to HFD control ($P = 0.02$). In additions levels in the urine were similar to that of the standard control animals (Figure 5.7A). However, no significant differences were seen in serum 8-isoprostane concentrations among the treatments.

8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, is the most frequently detected and studied DNA adduct. Upon DNA repair, 8-OHdG is excreted in the urine (Halliwell 2000). Urinary 8-OHdG is hypothesized not only to be a biomarker of generalized

cellular oxidative stress but might be a risk factor for cancer, atherosclerosis and diabetes (Wu, Chiou et al. 2004). In our study, urinary 8-OHDG concentrations followed a similar trend as 8-isoprostane. The HFD controls had the highest 8-OHDG concentrations suggesting elevated oxidative stress. All the potato diet consuming animals had suppressed 8-OHDG concentrations, to the levels as standard controls ($P = 0.01$, Figure 5.7A). We measured serum anti-oxidant status using ORAC method. We did not observe any differences in the hydrophilic and lipophilic serum ORAC levels among groups (Appendix VI).

5.5.4 Potato Diets Suppressed Systemic TNF-α and IL-1β in HFD Consuming Animals.

Elevated concentrations of circulating cytokines TNF-α, IL-6 and IL-1β are considered a risk factor for disorders such as insulin resistance and cardiovascular diseases (Wisse 2004, Berg and Scherer 2005). A growing body of evidence suggests that the pro-inflammatory cytokines IL-1β and TNF-α play a critical role in the pathogenesis of inflammatory diseases (Antonelli, Ferri et al. 2009). TNF-α and IL-1β share several pro-inflammatory properties including the promotion of leukocyte-endothelium interaction, the activation of the arachidonic acid pathway, and the induction of cytokine production (Antonelli, Ferri et al. 2009). In our study, serum TNF-α and IL-1β were suppressed in all the potato diet consuming animals compared to the HFD controls. Levels were similar to the standard controls suggesting that potato diets prevented HFD elevated systemic inflammatory markers (Figure 5.7B). We measured serum LPS, however, no differences were observed in serum LPS among the groups (Appendix VI).

Previous studies on potatoes have shown that potato peel extracts reduced hyperglycemia, oxidative stress, and overall food consumption in diabetic rodents when consumed at 10 % of the diet. Plasma glucose levels in the diabetic rats consuming potato peels were 33 % lower than the

control diabetic animals (Singh, Kamath et al. 2005, Singh, Kamath et al. 2005). Thus, we fasted the animals for 12 hours and collected serum to measure serum insulin/glucose. We did not observe any differences among groups in serum insulin or glucose. In fact, the levels of insulin in all groups were less than 5 mU/L which suggests that insulin levels were normal in these animals including the HFD controls (Figure 5.7B). To confirm, we measured expression of inflammatory markers NF-κB and TNF-α in the liver of these animals. We did not observe any differences among groups in the expression of NF-κB and TNF-α or the GSH:GSSG ratio in the livers of these animals (Appendix VII). In addition, we asked a trained pathologist to read the H&E slides of the livers of these animals. There were no signs of histological inflammation in the liver of these animals. Even after 13 weeks of HFD feeding, lack of liver inflammation might explain no systemic dysfunction in insulin and glucose levels. Thus, the current model – domestic pigs protected against insulin resistance even after 13 weeks of HFD feeding (Sejersen, Sorensen et al. 2013).

Rats consuming potato peels for 4 weeks showed lower plasma cholesterol (40 % decrease) and lower hepatic fat cholesterol (30 % decrease) compared with cellulose consuming rats (Lazarov and Werman 1996). Rats consuming a diet with 78 % potato for 3 weeks had lower plasma cholesterol and triglycerides and reduced liver cholesterol than control rats (Robert, Narcy et al. 2006). Therefore, we measured the lipid profile of serum in these animals. We did not see any significant differences within the HFD animals in total cholesterol, LDL and HDL levels. Levels of HDL and total cholesterol were higher in the HFD animals compared to the standard control animals (Figure 5.7C). Only the serum triglyceride levels were suppressed in the potato consuming animals compared to the HFD controls and were similar to that of the standard group (Figure 5.7C). Nevertheless, the lipid profile in these animals was in normal range

(Mersmann, Arakelian et al. 1979), even in the HFD controls, suggesting that there was no impairment in lipid metabolism in these animals. Lack of substantial differences between the standard control and the high-fat diet fed control animals suggests that the pig model used in the study is protected against high-fat diet induced dyslipidemia and insulin resistance in the time period employed (13 weeks). However, recently published research indicates that the young Göttingen minipig fed on a high-energy diet for 4 months (~ 16-17 weeks) is a better model for diet induced childhood/adolescent obesity and metabolic syndrome (Christoffersen, Golozoubova et al. 2013), thus future studies focusing on preventing HFD induced insulin resistance and dyslipidemia should focus on Göttingen minipigs as a model.

5.6 Conclusions

In conclusion, our study suggests that both purple and white-fleshed potatoes demonstrate anti-oxidant/anti-inflammatory properties – processes involved in progression of colon inflammation/cancer and other disorders. Consumption of purple-fleshed potato demonstrated only a marginal benefit over white-fleshed potatoes. However, our earlier reversal study revealed that only the consumption of purple-fleshed potato reversed detrimental effects of HFD in pigs. Results from both studies demonstrate that only the consumption of purple-fleshed potatoes could both prevent and reverse HFD elevated oxidative stress and inflammatory markers in HFD consuming pigs.

Regardless of the health-benefits, the sensory attributes and consumer acceptance of these new color-fleshed cultivars should not be discounted. Earlier results on sensory analysis from our lab revealed consumers' readiness to accept purple-fleshed potatoes provided they were educated on the health benefits (Madiwale, Reddivari et al. 2011, Madiwale, Reddivari et al. 2012).

Hence, purple-fleshed potatoes can be a healthier choice as they possess greater levels of bioactive compounds, even after processing, as compared with their white-fleshed counterparts. However, we should keep in mind that the potato should be a healthy component of a varied diet. As a starch food item, it should be consumed in moderation and without excess lipid additions. Potato servings do not in themselves promote obesity/related disorders; excess starchy food, refined sugar and food laden with high calorie lipid additions might be the culprit (Brown 2005, Camire, Kubow et al. 2009).

5.7 References

Antonelli, A., C. Ferri, S. M. Ferrari, E. Ghiri, F. Goglia, A. Pampana, F. Bruschi and P. Fallahi (2009). "Serum levels of proinflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor alpha in mixed cryoglobulinemia." Arthritis Rheum **60**(12): 3841-3847.

Autism, A. o. (2011). Johns Hopkins Reports More Children Have Inflammatory Bowel Disease. Age of Autism.

Berg, A. H. and P. E. Scherer (2005). "Adipose tissue, inflammation, and cardiovascular disease." Circ Res **96**(9): 939-949.

Brown, C. R. (2005). "Antioxidants in potato." Am J Potato Res **82**: 163-172.

Camire, M. E., S. Kubow and D. J. Donnelly (2009). "Potatoes and Human Health." Crit Rev Food Sci Nutr **49**(10): 823-840.

Carcamo, J. M., A. Pedraza, O. Borquez-Ojeda and D. W. Golde (2002). "Vitamin C suppresses TNF alpha-induced NF kappa B activation by inhibiting I kappa B alpha phosphorylation." Biochem **41**(43): 12995-13002.

Christoffersen, B., V. Golozoubova, G. Pacini, O. Svendsen and K. Raun (2013). "The young Gottingen minipig as a model of childhood and adolescent obesity: influence of diet and gender." Obesity **21**(1): 149-158.

Chuang, C. C. and M. K. McIntosh (2011). "Potential mechanisms by which polyphenol-rich grapes prevent obesity-mediated inflammation and metabolic diseases." Annu Rev Nutr **31**: 155-176.

Cooper, D. A., D. A. Berry, V. A. Spendel, A. L. Kiorpes and J. C. Peters (1997). "The domestic pig as a model for evaluating olestra's nutritional effects." J Nutr **127**(8 Suppl): 1555S-1565S.

Dewanto, V., X. Wu, K. Adom and R. Liu (2002). "Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity." J Agric Food Chem **50**(10): 3010-3014.

Ding, S., M. M. Chi, B. P. Scull, R. Rigby, N. M. Schwerbrock, S. Magness, C. Jobin and P. K. Lund (2010). "High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse." PLoS One **5**(8): e12191.

FAO. (2009). "FAO Statistical Databases." Retrieved 25th January 2011, from http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor.

Fernandez-Sanchez, A., E. Madrigal-Santillan, M. Bautista, J. Esquivel-Soto, A. Morales-Gonzalez, C. Esquivel-Chirino, I. Durante-Montiel, G. Sanchez-Rivera, C. Valadez-Vega and J. A. Morales-Gonzalez (2011). "Inflammation, oxidative stress, and obesity." Int J Mol Sci **12**(5): 3117-3132.

Gambero, A., M. Marostica, M. J. Abdalla Saad and J. Pedrazzoli, Jr. (2007). "Mesenteric adipose tissue alterations resulting from experimental reactivated colitis." Inflamm Bowel Dis **13**(11): 1357-1364.

Halliwell, B. (2000). "Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come?" Am J Clinical Nutr **72**(5): 1082-1087.

Han, J. C., D. A. Lawlor and S. Y. S. Kimm (2010). "Childhood obesity." Lancet **375**(9727): 1737-1748.

Hou, J. K., B. Abraham and H. El-Serag (2011). "Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature." Am J Gastroenterol **106**(4): 563-573.

Huang, D., B. Ou, M. Hampsch-Woodill, J. A. Flanagan and R. L. Prior (2002). "High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format." J Agric Food Chem **50**(16): 4437-4444.

Karlsen, A., L. Retterstol, P. Laake, I. Paur, S. K. Bohn, L. Sandvik and R. Blomhoff (2007). "Anthocyanins inhibit nuclear factor-kappaB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults." J Nutr. **137**(8): 1951-1954.

Khosravi-Boroujeni, H., N. Mohammadifard, N. Sarrafzadegan, F. Sajjadi, M. Maghroun, A. Khosravi, H. Alikhasi, M. Rafieian and L. Azadbakht (2012). "Potato consumption and cardiovascular disease risk factors among Iranian population." Int J Food Sci Nutr **63**(8): 913-920.

Kim, K. A., W. Gu, I. A. Lee, E. H. Joh and D. H. Kim (2012). "High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway." PLoS One **7**(10): e47713.

Lam, Y. Y., A. J. Mitchell, A. J. Holmes, G. S. Denyer, A. Gummesson, I. D. Caterson, N. H. Hunt and L. H. Storlien (2011). "Role of the gut in visceral fat inflammation and metabolic disorders." Obesity **19**(11): 2113-2120.

Lazarov, K. and M. J. Werman (1996). "Hypocholesterolaemic effect of potato peel as a dietary fibre source." Med Sci Res **24**: 581-582.

Li, H., C. Lelliott, P. Hakansson, K. Ploj, A. Tuneld, M. Verolin-Johansson, L. Benthem, B. Carlsson, L. Storlien and E. Michaelsson (2008). "Intestinal, adipose, and liver inflammation in diet-induced obese mice." Metabolism **57**(12): 1704-1710.

Lin, B.-H. and R. M. Morrison (2002). "Higher Fruit Consumption Linked With Lower Body Mass Index (Economic Research Service, USDA)." Food Rev **25**(3): 28-32.

Luceri, C., F. Guglielmi, M. Lodovici, L. Giannini, L. Messerini and P. Dolara (2004). "Plant phenolic 4-coumaric acid protects against intestinal inflammation in rats." Scand J Gastroenterol **39**(11): 1128-1133.

Madiwale, G. P., L. Reddivari, D. G. Holm and J. Vanamala (2011). "Storage elevates phenolic content and antioxidant activity but suppresses antiproliferative and pro-apoptotic properties of colored-flesh potatoes against human colon cancer cell lines." J Agric Food Chem **59**(15): 8155-8166.

Madiwale, G. P., L. Reddivari, M. Stone, D. G. Holm and J. Vanamala (2012). "Combined effects of storage and processing on the bioactive compounds and pro-apoptotic properties of color-fleshed potatoes in human colon cancer cells." J Agric Food Chem **60**(44): 11088-11096.

Matsuzawa-Nagata, N., T. Takamura, H. Ando, S. Nakamura, S. Kurita, H. Misu, T. Ota, M. Yokoyama, M. Honda, K. Miyamoto and S. Kaneko (2008). "Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity." Metabolism **57**(8): 1071-1077.

Mersmann, H. J., M. C. Arakelian and L. J. Brown (1979). "Plasma lipids in neonatal and growing swine." J Anim Sci **48**(3): 554-558.

Monaco, C. and E. Paleolog (2004). "Nuclear factor kappaB: a potential therapeutic target in atherosclerosis and thrombosis." Cardiovasc Res **61**(4): 671-682.

Montuschi, P., P. J. Barnes and L. J. Roberts, 2nd (2004). "Isoprostanes: markers and mediators of oxidative stress." FASEB J **18**(15): 1791-1800.

Nicoli, M., M. Anese and M. Parpinel (1999). "Influence of processing on the antioxidant properties of fruit and vegetables." Trends Food Sci Tech **10**(3): 94-100.

NPC (2007). Potato statistical yearbook, National Potato Council (NPC).

Owen, J. B. and D. A. Butterfield (2010). "Measurement of oxidized/reduced glutathione ratio." Methods Mol Biol **648**: 269-277.

Piberger, H., A. Oehme, C. Hofmann, A. Dreiseitel, P. G. Sand, F. Obermeier, J. Schoelmerich, P. Schreier, G. Krammer and G. Rogler (2011). "Bilberries and their anthocyanins ameliorate experimental colitis." Mol Nutr Food Res **55**(11): 1724-1729.

Pond, W. G. and X. G. Lei (2000). Of pigs and people. Swine Nutrition. Washington, D.C., CRC Press**:** 3-18.

Price, K., J. Bacon and M. Rhodes (1997). "Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*)." J Agric Food Chem **45**(3): 938-942.

Radhakrishnan, S., L. Reddivari, G. Madiwale, S. W. Kim and J. Vanamala (2011). "Purple potato anthocyanins exert anti-cancer properties and suppress oxidative stress even after processing." Potato Association of America - PAA 2011.

Robert, L., A. Narcy, E. Rock, C. Demigne, A. Mazur and C. Remesy (2006). "Entire potato consumption improves lipid metabolism and antioxidant status in cholesterol-fed rat." Eur J Nutr **45**(5): 267-274.

Rodriguez-Ramiro, I., S. Ramos, E. Lopez-Oliva, A. Agis-Torres, L. Bravo, L. Goya and M. A. Martin (2013). "Cocoa polyphenols prevent inflammation in the colon of azoxymethane-treated rats and in TNF-alpha-stimulated Caco-2 cells." Br J Nutr **110**(2): 206-215.

Rodriguez-Saona, L. E., M. M. Giusti and R. E. Wrolstad (1998). "Anthocyanin pigment composition of red-fleshed potatoes." J Food Sci **63**(3): 458-465.

Schulze-Osthoff, K., M. K. Bauer, M. Vogt and S. Wesselborg (1997). "Oxidative stress and signal transduction." Int J Vitam Nutr Res **67**(5): 336-342.

Sejersen, H., M. T. Sorensen, T. Larsen, E. Bendixen and K. L. Ingvartsen (2013). "Liver protein expression in young pigs in response to a high-fat diet and diet restriction." J Anim Sci **91**(1): 147-158.

Singh, N., V. Kamath and P. S. Rajini (2005). "Attenuation of hyperglycemia and associated biochemical parameters in STZ-induced diabetic rats by dietary supplementation of potato peel powder." Clin Chim Acta **353**(1-2): 165-175.

Singh, N., V. Kamath and P. S. Rajini (2005). "Protective effect of potato peel powder in ameliorating oxidative stress in streptozotocin diabetic rats." Plant Foods Hum Nutr **60**(2): 49-54.

Spanos, G., R. Wrolstad and D. Heatherbell (1990). "Influence of processing and storage on the phenolic composition of apple juice." J Agric Food Chem **38**(7): 1572-1579.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol **16**(1): 3-9.

Teixeira, L. G., A. J. Leonel, E. C. Aguilar, N. V. Batista, A. C. Alves, C. C. Coimbra, A. V. Ferreira, A. M. de Faria, D. C. Cara and J. I. Alvarez Leite (2011). "The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation." Lipids Health Dis **10**: 204.

Walton, M. C., R. G. Lentle, G. W. Reynolds, M. C. Kruger and T. K. McGhie (2006). "Anthocyanin absorption and antioxidant status in pigs." J Agric Food Chem **54**(20): 7940-7946.

Wisse, B. E. (2004). "The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity." J Am Soc Nephrol **15**(11): 2792-2800.

Wu, L. H., Z. L. Xu, D. Dong, S. A. He and H. Yu (2011). "Protective Effect of Anthocyanins Extract from Blueberry on TNBS-Induced IBD Model of Mice." Evid Based Complement Alternat Med **2011**: 525462.

Wu, L. L., C. C. Chiou, P. Y. Chang and J. T. Wu (2004). "Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics." Clinica Chimica Acta **339**(1-2): 1-9.

Yilmaz, Ö., S. Keser, M. Tuzcu, M. Güvenc, B. Çetintas, S. Irtegün, H. Tastan and K. Sahin (2009). "A Practical HPLC Method to Measure Reduced (GSH) and Oxidized (GSSG) Glutathione Concentrations in Animal Tissues." J Anim Vet Adv **8**(2): 343-347.

Zhang, C., X. Xu, B. J. Potter, W. Wang, L. Kuo, L. Michael, G. J. Bagby and W. M. Chilian (2006). "TNF-alpha contributes to endothelial dysfunction in ischemia/reperfusion injury." Arterioscler Thromb Vasc Biol **26**(3): 475-480.

CHAPTER VI

SUMMARY AND CONCLUSIONS

6.1 *In vitro* Study

Our results demonstrated that suppression of proliferation and elevation of apoptosis in HCT-116 p53+/+ human colon cancer cell lines at 100 µM or higher concentrations of RSV could be accomplished at 25 µM, when combined with GSE at doses of 35-50 µg/ml. These results strongly support our hypothesis that combining bioactive compounds like RSV and GSE could reduce the dose of either compound while providing similar or better anti-colon cancer properties. This lends support to the proposal that combinatorial approach towards colon cancer chemoprevention using bioactive compounds is a feasible approach – however, *in vivo* studies of such a RSV-GSE combination are clearly warranted.

RSV-GSE combination induced G_0/G_1 phase arrest in HCT-116 colon cancer cells. RSV potentiated GSE induced p53 dependent apoptosis via mitochondrial apoptotic signaling as it involved interplay of reactive oxygen species, p53, Bax/Bcl-2 and caspase-3 pathways. Combination of RSV and GSE suppressed proliferation and induced apoptosis even in presence of IGF-1, suggesting its potential as a chemopreventive agent against colon cancer even in obese subjects. Moreover, RSV-GSE demonstrated specificity as the combination did not alter cell kinetics in the normal colonic epithelial cell line CRL-1831. Given the superior *in vitro* activity, further *in vivo* confirmation, using spontaneous or sporadic colon cancer mice models, of the potential effects of such combination as an anti-tumorigenic agent is clearly required.

6.2 Animal Study

In the prevention study, both purple and white-fleshed potato (raw, baked and chips) consuming animals had lower distal colonic oxidative stress (measured using GSH:GSSG ratio, significant for purple-fleshed potato raw group) and significantly lower distal colonic expression of innate pro-inflammatory markers including TLRs 2, 4, NF-κB, TNF-α, and COX-2, compared to the high-fat diet (HFD) controls ($P < 0.05$). The levels in the animals consuming potato diets were similar to the standard control animals. Similarly, in the mesenteric fat, oxidative stress (GSH:GSSG ratio) was lower (only numerical suppression in WB and WC groups), and expression levels of TLR-4, NF-κB and TNF-α were significantly suppressed in all the potato diet consuming animals compared to the HFD controls. Moreover, the animals consuming potato diets had significantly lower systemic oxidative stress markers (measured using urinary 8-isoprostane and 8-OHDG) and circulating pro-inflammatory cytokines (TNF-α and IL-1β) compared to the HFD control animals similar to levels observed in the standard control animals ($P < 0.05$).

However, in the reversal study, only the purple-fleshed potato consuming animals had significantly lower distal colonic oxidative stress (GSH:GSSG ratio) and lower distal colonic expression of innate inflammatory markers (TLRs 2, 4, NF-κB, and TNF-α) compared to HFD controls. Levels of these markers in the animals consuming white-fleshed potatoes were not significantly different from the HFD controls. Only the animals consuming purple-fleshed potato diets had lower expression of TNF-α and higher expression of adiponectin in the mesenteric fat compared to the HFD control. Anti-inflammatory/anti-oxidant effect of the purple-fleshed potato in the mesenteric fat was only modest in the reversal study. The purple-fleshed potato consuming animals had significantly lower serum 8-isoprostane compared to the HFD control, levels in the

white-fleshed potato group were not significantly different from HFD control. Serum TNF-α, a pro-inflammatory cytokine, however, was suppressed in both purple and white-fleshed groups (W10) compared to the HFD control.

Results of our study demonstrate that only purple-fleshed potato, even after processing, prevented and reversed HFD induced oxidative stress/inflammation markers in the distal colon, mesenteric fat and systemic circulation in the pig, a human relevant animal model. The differences between the purple and white-fleshed potatoes could be explained by the differences in their phenolic metabolic profiles as identified using UPLC/MS analysis. However, white-fleshed potato (rich in phenolic acids) was effective in preventing HFD elevated oxidative stress/inflammation markers in the distal colon, mesenteric fat and systemic circulation. Vitamin C, phenolic acids and resistant starches present in the white-fleshed potatoes could have mediated the health benefits, however further research to elucidate their contributions is required. Overall results suggest that purple-fleshed potatoes are a great delivery vehicle for anti-inflammatory and anti-oxidant bioactive compounds – may potentially reduce risk for systemic and colonic inflammatory disorders.

APPENDIX I

Growth performances (feed conversion ratio and back-fat thickness in mm) of pigs consuming experimental diets during the 5 week feeding period in the reversal study.

Dietary Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	
				Feed Conversion Ratio			
C		0.3 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.4 ± 0	0.3 ± 0
P10		0.4 ± 0	0.2 ± 0.1	0.4 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
P20		-0.7 ± 0.6	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0	0.3 ± 0
W10		0.3 ± 0.1	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
W20		-1.3 ± 1.1	0.1 ± 0.2	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0
				Back-fat Thickness (mm)			
C	18.8 ± 0.2	17.2 ± 0.2	18.6 ± 0.2	18.5 ± 0.2			
P10	18.7 ± 0.3	17.7 ± 0.1	18.5 ± 0.3	18.5 ± 0.3			
P20	18.8 ± 0.3	17.3 ± 0.3	18.7 ± 0.3	18.6 ± 0.3			
W10	18.4 ± 0.1	17.7 ± 0.3	18.3 ± 0.1	18.2 ± 0.1			
W20	18.8 ± 0.2	17.5 ± 0.2	18.6 ± 0.2	18.5 ± 0.2			

Data are presented as means ± S.E. C: High-fat diet control, P10 and P20 - purple-fleshed potato chips at 10 and 20 % of diet w/w and W10 and W20 - white-fleshed potato chips at 10 and 20 % of the diet w/w. There were no differences between groups at $P \leq 0.05$.

APPENDIX II

Oxidative stress and inflammatory markers measured in porcine serum/urine at week 5 in the reversal study.

Markers/Parameters	C	P10	P20	W10	W20
Serum total oxygen radical absorbance capacity (μmol Trolox equivalents/L)	906.6 ± 45.2[a]	867.1 ± 46.2[a]	843.1 ± 43.2[a]	806.0 ± 28.7[a]	844.0 ± 42.3[a]
Serum C-reactive protein (μg/mL)	11.2 ± 1.8[a]	10.7 ±1.2[a]	13.9 ±1.9[a]	11.9 ±3.2[a]	13.4 ±1.8[a]
Serum lipopolysaccharide (ng/mL)	0.09 ± 0.02[a]	0.11 ± 0.05[a]	0.12 ± 0.04[a]	0.09 ± 0.03[a]	0.1 ± 0.04[a]
Serum leukotriene B4 (pg/mL)	2390.0 ± 330.2[ab]	2054.2 ± 87.1[a]	2036.2 ± 153.7[a]	2117.1 ± 215.3[ab]	2573.1 ± 228.4[b]
Urinary 8-hydroxy-2′-deoxyguanosine (ng/mg creatinine)	51.8 ± 8.9[a]	59.8 ± 10.3[a]	45.5 ± 5.9[a]	57.4 ± 9.4[a]	42.5 ± 6.0[a]
Urinary 8-isoprostane (ng/mg creatinine)	0.9 ± 0.1[a]	1.2 ± 0.3[a]	1.3 ± 0.3[a]	1.1 ± 0.2[a]	1.2 ± 0.2[a]

Data are presented as means ± S.E. Means in a row that differ by a common letter (a, b) differ ($P < 0.05$). C: High-fat diet control, P10 and P20 - purple-fleshed potato chips at 10 and 20 % of diet w/w and W10 and W20 - white-fleshed potato chips at 10 and 20 % of the diet w/w.

APPENDIX III

Relative expression as measured using real time PCR in the ileum and the proximal colon mucosa in the reversal study.

	TLR-4	**NF-κB**	**TNF-α**
Dietary Group		**Ileum Mucosa**	
C	1.9 ± 0.8	1.4 ± 0.4	1.6 ± 0.3
P10	1.3 ± 0.7	1 ± 0.4	1 ± 0.3
P20	2 ± 0.6	1.2 ± 0.4	0.9 ± 0.3
W10	2.6 ± 0.6	1.4 ± 0.4	1.5 ± 0.3
W20	1.5 ± 0.6	1 ± 0.4	1.3 ± 0.3
		Proximal Colon Mucosa	
C	0.9 ± 0.1	1.2 ± 0.2	0.9 ± 0.2
P10	0.8 ± 0.1	0.8 ± 0.2	0.6 ± 0.3
P20	0.9 ± 0.1	0.9 ± 0.2	0.6 ± 0.2
W10	0.7 ± 0.1	0.6 ± 0.4	0.5 ± 0.2
W20	0.8 ± 0.1	0.9 ± 0.2	1.4 ± 0.8

Results were expressed as mean ± S.E. for 7-8 animals in each treatment group. C: High-fat diet control, P10 and P20 - purple-fleshed potato chips at 10 and 20 % of diet w/w and W10 and W20 - white-fleshed potato chips at 10 and 20 % of the diet w/w. There were no differences between groups at $P \leq 0.05$.

APPENDIX IV

Feed conversion ratio of animals in the prevention study. Data are presented as means ± S.E of 8 animals per group.

Dietary group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Overall FCR
	Feed Conversion Ratio (FCR)														
C1			1.3 ± 0.2	4 ± 0.3	1.4 ± 0	1.9 ± 0.1	2 ± 0.2	2 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	2.5 ± 0.2	2.2 ± 0
C2			1 ± 0	4.3 ± 0.3	1.4 ± 0	1.9 ± 0.1	1.7 ± 0.1	2 ± 0.1	2 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.5 ± 0.2	2.1 ± 0
PR			1 ± 0	4.5 ± 0.9	1.2 ± 0	2.1 ± 0.1	1.3 ± 0	2.5 ± 0.2	1.9 ± 0	2 ± 0	1.5 ± 0.1	2.8 ± 0.4	2 ± 0.1	4.6 ± 1.2	2.1 ± 0
WR			1 ± 0	3.6 ± 0.3	1.2 ± 0.1	2.6 ± 0.2	1.3 ± 0	2.4 ± 0.1	2 ± 0	2.1 ± 0.1	1.9 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	2.7 ± 0.2	2.2 ± 0.1
PB			1 ± 0	3.5 ± 0.4	1.3 ± 0.1	2.1 ± 0.1	1.5 ± 0.1	2.4 ± 0.1	1.8 ± 0.1	2.2 ± 0.1	1.6 ± 0.1	2.7 ± 0.2	2.1 ± 0.1	3 ± 0.2	2.1 ± 0
WB			1 ± 0	4 ± 0.2	1.3 ± 0.1	2.4 ± 0.1	1.4 ± 0.1	2.6 ± 0.1	1.9 ± 0.1	2.5 ± 0.2	2.3 ± 0.2	2 ± 0.1	2 ± 0.1	4.8 ± 1	2.1 ± 0

PC	1 ± 0	4.7 ± 0.8	1.2 ± 0	2.3 ± 0.1	1.4 ± 0.1	2.5 ± 0.2	1.8 ± 0.1	2.1 ± 0.1	1.5 ± 0.1	2.9 ± 0.2	1.9 ± 0.1	3.4 ± 0.5	2.2 ± 0.1
WC	1 ± 0.1	3.7 ± 0.3	1.2 ± 0	2.5 ± 0.2	1.3 ± 0	2.9 ± 0.3	1.9 ± 0.1	2.2 ± 0.1	2 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	3 ± 0.2	1.9 ± 0.2

Data are presented as means ± S.E. C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation. There were no differences between groups in the overall feed conversion ratio at $P \leq 0.05$. FCR: Feed conversion ratio.

APPENDIX V

Back-fat thickness (mm) of animals in the prevention study. Data are presented as means ± S.E of 8 animals per group.

Dietary Group	Week 1	Week 5	Week 8	Week 13
	Back-fat Thickness (inch)			
C1	7 ± 0.5	8.8 ± 0.5	12.4 ± 0.6	19.2 ± 1
C2	7.2 ± 0.7	11.2 ± 0.8	14.4 ± 1	19.1 ± 1.3
PR	7.4 ± 0.7	10.2 ± 0.7	14.7 ± 0.9	18.6 ± 1.4
WR	8.1 ± 0.4	12.4 ± 0.7	16.5 ± 0.5	21.6 ± 1.2
PB	7.2 ± 0.5	11.4 ± 0.6	16.5 ± 0.7	23.3 ± 0.6
WB	7.3 ± 0.5	10.9 ± 1	15.5 ± 1.1	20.8 ± 1.9
PC	7.1 ± 0.4	12.1 ± 0.7	17.1 ± 0.8	24.7 ± 1.5
WC	7 ± 0.3	12.9 ± 1.1	18.8 ± 1.1	23.1 ± 1.9

Data are presented as means ± S.E. There were no differences between groups at $P \leq 0.05$. C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation. There were no differences between groups at $P \leq 0.05$.

APPENDIX VI

Oxidative stress and inflammatory markers measured in porcine serum/urine at week 5 in the prevention study.

Markers/Parameters	C1	C2	PR	WR	PB	WB	PC	WC
Serum lipopolysacccharide (ng/mL)	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0
Serum 8-isoprostane (pg/ml)	507.05 ± 47.31	711.04 ± 116.03	502.21 ± 118.28	791.37 ± 105.56	786.58 ± 88.04	609.48 ± 139.84	511.29 ± 97.95	633.08 ± 147.8
Serum total oxygen radical absorbance capacity (µmol Trolox equivalents/L)	487.8 ± 28.8	437.1 ± 38.4	531.7 ± 77.8	504.9 ± 66.9	446.7 ± 71	440.1 ± 44.8	493.3 ± 55.9	604.9 ± 66.9

Data are presented as means ± S.E. C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation. There were no differences between groups at P ≤ 0.05.

APPENDIX VII

GSH:GSSG ratio in the liver was measured using UPLC/PDA. Relative expression of TLR-4, NF-κB and TNF-α was measured using real-time PCR in the liver, ileum and the proximal colon mucosa in the prevention study.

	GSH:GSSG Ratio	**NF-κB**	**TNF-α**
Dietary Group		**Liver**	
C1	3.6 ± 0.3	1.3 ± 0.3	1.4 ± 0.5
C2	3.7 ± 0.1	1.1 ± 0.3	1.2 ± 0.4
PR	3.5 ± 0.4	0.9 ± 0.3	2.2 ± 0.6
WR	3.3 ± 0.2	0.8 ± 0.3	1.1 ± 0.5
PB	3.5 ± 0.3	1.4 ± 0.3	1.9 ± 1.5
WB	4 ± 0.3	0.9 ± 0.3	2.1 ± 1.4
PC	3.6 ± 0.2	1 ± 0.3	0.8 ± 0.5
WC	3.3 ± 0.1	0.9 ± 0.3	1.2 ± 1
	TLR-4	**NF-κB**	**TNF-α**
		Ileum Mucosa	
C1	1.3 ± 0.3	1.9 ± 0.3	0.5 ± 0.4
C2	1 ± 0.3	1 ± 0.3	0.5 ± 0.4
PR	1.7 ± 0.3	1.5 ± 0.3	1.2 ± 0.5
WR	0.9 ± 0.3	1 ± 0.3	0.4 ± 0.4

PB	0.6 ± 0.3	0.8 ± 0.3	0.6 ± 0.4
WB	1.5 ± 0.3	1.6 ± 0.3	0.4 ± 0.5
PC	1.1 ± 0.3	1 ± 0.3	1.1 ± 0.4
WC	1 ± 0.3	1.3 ± 0.3	1 ± 0.5
	TLR-4	**NF-κB**	**TNF-α**
	Proximal Colon Mucosa		
C1	0.6 ± 0.5	0.8 ± 0.4	0.7 ± 0.2
C2	0.6 ± 0.6	0.5 ± 0.5	1.2 ± 0.5
PR	1.2 ± 0.5	1.2 ± 0.4	0.9 ± 0.3
WR	0.5 ± 0.5	0.5 ± 0.4	1.1 ± 0.3
PB	1 ± 0.5	0.9 ± 0.4	0.5 ± 0.4
WB	0.5 ± 0.5	0.5 ± 0.4	0.4 ± 0.4
PC	0.4 ± 0.5	0.4 ± 0.4	0.5 ± 0.3
WC	0.6 ± 0.5	0.5 ± 0.4	0.9 ± 0.3

Data are presented as means ± S.E. C1, standard control, C2, high-fat diet control, letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10 % supplementation. There were no differences between groups at $P \leq 0.05$.

LIST OF ABBREVIATIONS

5-FU: 5-florouracil

8-OHDG: 8-hydroxydeoxyguanosine

Akt: Protein Kinase B

APC: Adenomatous polyposis coli

ATM: Adipose tissue macrophages

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2 protein

CD: Crohn’s disease

CI: Combination index

COX-2: Cyclooxygenase-2

CRC: Colorectal cancer

DMEM: Dulbecco's modified eagle's medium

FACS: Fluorescence activated cell sorting analysis

FBS: Fetal bovine serum

GSE: Grape seed extract

GSH: Reduced glutathione

GSSG: Oxidized glutathione

HDL: High density lipoprotein

HFD: High-fat diet

IBD: Inflammatory bowel disease

IFN-γ: Interferon- γ

IGF-1: Insulin like growth factor -1

IGF-1R: Insulin like growth factor-1 receptor

IGFBP: Insulin like growth factor binding protein

IL-10: Interleukin-10

IL-1β: Interleukin-1β

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

JNK: c-Jun N-terminal kinases

LDH: Lactate-dehydrogenase

LDL: Low density lipoprotein

LPS: Lipopolysaccharide

MCP-1: Monocyte chemotactic protein-1

MDA: Malondialdehyde

NAC: N-Acetyl cysteine

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate buffered saline

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RSV: Resveratrol

TGF-β: Transforming growth factor-β

TLR-2: Toll-like receptor-2

TLR-4: Toll-like receptor-4

TNF-α: Tumor necrosis factor-α

UC: Ulcerative colitis

UPLC-MS: Ultra performance liquid chromatography – Mass spectrometry

REVERSAL STUDY

C: High-fat diet control

P10 and P20: Purple-fleshed potato chips at 10 and 20 % of diet w/w.

W10 and W20: White-fleshed potato chips at 10 and 20 % of the diet w/w.

PREVENTION STUDY

C1: Standard diet control

C2: High-fat diet control,

PR, PB, PC, WR, WB and WC: Letters P and W indicate purple and white-fleshed potato groups, R, B & C indicate processing treatments raw, baked and chips, respectively at 10% supplementation (w/w).