An Obese Genotype Affects Apoptosis Related Gene Expression

by

Nafiseh Nafissi

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2008

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Apoptosis is a genetically regulated form of cell death that occurs when the cell is exposed to physiological, pathogenic, or cytotoxic stimuli. Unregulated apoptosis (too much or too little apoptosis) at any time from embryogenesis to adulthood, can result in a variety of disease states, such as neurodegenerative disorders, autoimmunity, cardiovascular disease, liver and kidney problems, and cancer. A reasonable estimation is that either too little or too much cell death contributes to half of the main medical illnesses for which adequate therapy or prevention is lacking. The apoptotic pathways can be initiated by reactive oxygen species (ROS) and inflammatory molecules, both of which are believed to be up-regulated in a state of obesity. In addition, multiple studies have shown that the risk of developing cardiovascular disease, type 2 diabetes mellitus, nonalcoholic fatty liver disease, and certain types of cancers increase with increasing degree of obesity in both men and women. Despite the well characterized association of obesity and disease incidence, the mechanisms by which obesity contributes to disease pathology are poorly understood. Previously, in our research group, it was shown that obese Zucker rats, which are the animal model of human obesity, are more prone to colon cancer and hepatic steatosis compare to their relative lean counterparts. Therefore, applying Real-Time RT-PCR, the expression levels of some pro- and anti-apoptotic members of the BCL-2 family of genes were investigated to figure out the possible effect of obesity on apoptotic gene expression levels. Also, apoptotic gene expression patterns of obese and lean Zucker rats after DNA damage induction were compared to each other in order to find the possible connection of apoptotic gene expression with disease progression in obese individuals. This is the first study comparing the expression level of BCL-2 family of genes in obese versus lean liver and colon tissue. In this study, it was shown that an obese genotype affects pro- and anti-apoptotic gene expression levels and patterns whether or not DNA damage has been induced in both liver and colon. The results show a clear alteration in apoptotic gene expression levels in obese individuals compared to their lean counterparts leading to the proposal that apoptosis may be involved in the obesity related colon cancer and liver steatosis.

Acknowledgements

I achieve nothing without the grace of God, the devotion from my teachers, and the support of my family and friends

I would like to express my appreciation to Professor Ranjana Bird, who gave me the opportunity of being her Master's student. It was very auspicious that she opened her lab to me and gave me the chance to work with her when other doors were closed. I am especially grateful for her trust on me.

It is with great pleasure that I also thank my co-supervisor, Professor Brian Dixon. Words cannot express the extent of my gratitude for his invaluable guidance and advice while I was writing my thesis. I was very fortunate that he took me on as a student.

I gratefully acknowledge my committee members, Professor Matt Vijayan and Dr. Mungo Marsden, for their advice and guidance. I would like to especially thank Matt, who kindly provided the Real-Time PCR machine used in this research project, and for his constructive comments on my thesis.

I also deeply appreciate the help and support of Dr. Dragana Miskovic. I made several visits to her office to discuss research problems or ask her opinion about a decision I had to make. Her advice has nourished my intellectual maturity, from which I will continue to benefit.

I would like to express my profound gratitude to my friend and previous colleague, Ramin Sarrami-Forooshani. His constructive guidance and criticism improved this thesis to a great extent. I am grateful in every possible way for his help, and I hope we can collaborate in the future as well.

To all my lab colleagues, thanks for your friendship! I would especially like to thank my reliable and true friend Sheva Naahidi, who always wants the best for me in my academic and personal life. I am grateful for the advice and kindness she showed me when I first joined the lab. Kate, thanks for all the great conversations and shared laughter.

To my light during the dark nights and my dearest teachers in life, my parents, Fatemeh Akrami and Professor Gholam-Reza Nafissi – how would I ever accomplish anything without their unconditional love and support? I am forever highly indebted to these pillars of my being. I would also like to acknowledge my elder brother and sisters, Hamid-Reza, Zohreh, and Nahid, for their love and support. I wish to express my very special thanks to my brother-in-law, Dr. Sirous Zeinali. I am deeply indebted to him for both my career and academic life.

Last, but most important, is my grateful appreciation to the best friend I ever had, my beloved husband Seyed-Mohammad Eram, who has been my guide on this difficult journey. Without his continuous love, support, and encouragement, this success would have been very difficult to achieve. And I would like to thank my dear son, Omid, for his love and, of course, his little distractions, which are cute and charming.

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

1.1 Apoptosis

Apoptosis — the regulated death of a cell — is a complicated process. The decision to die cannot be taken easily, and the activities of many genes influence a cell's fate by playing a role in its self-destruction programme. Once the decision is made by thd organism, proper execution of the apoptotic programme is essential for the coordinated activation and execution of multiple sub-programmes. Multi-cellular organisms often need to get rid of excess cells, regarding this purpose; they use an organized molecular program. As important as cell division and cell migration, regulated cell death allows the organism to tightly control cell numbers and tissue size, and to establish homeostasis. Programmed cell death acquired a number of names over the past two centuries (*1*). The term apoptosis finally adopted by Currie and co-workers in 1972 to describe a common type of programmed cell death that they repeatedly observed in various tissues and cell types (*2*). They noticed that these dying cells shared many morphological features, which were distinct from the features observed in cells undergoing pathological and necrotic cell death, and they suggested that these shared morphological features should be the result of a common and conserved cell death programme (*3*). Apoptosis can now be defined not only by morphology, but also by molecular and biochemical mechanisms. Studies within the past few years have revealed that a complex group of molecules that make up the “the death machinery” regulates apoptosis. This pathway can be explained as an active process of cellular self-destruction with distinctive morphological and biochemical features (*4*). Two major apoptotic pathways have been defined in mammalian cells: the death-receptor-mediated or the extrinsic apoptotic pathway and the mitochondrial or the intrinsic apoptotic pathway. The extrinsic pathway can be activated through cell surface receptors by external stimuli such as growth factor withdrawal, UV or γ radiation, chemotherapy agents, free oxygen radicals, and heat shock leading to activation of cysteine-aspartic-acid-proteases (Caspases) (*5*). The intrinsic pathway is activated through mitochondrion as a result of cellular stress and DNA damage (Fig.1). In

this pathway, the BCL-2 family of genes and proteins are perhaps the most important regulators. These proteins can also be responsible for bridging signals from the death-receptor pathway to the mitochondrial pathway.

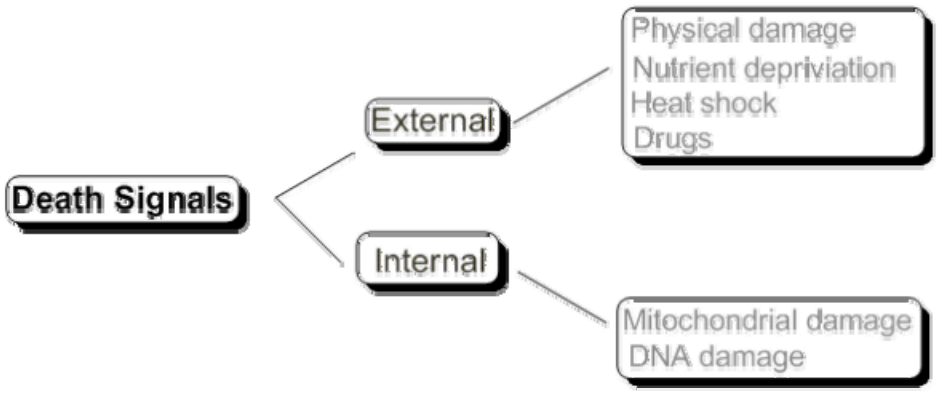


Figure 1 Death signals

Modified from (*6*)

BCL-2 family consists of both anti-apoptosis and pro-apoptosis members. While the pro-apoptosis members function as sensors for death signals and executors of the death program, the anti-apoptosis members inhibit the initiation of the death program. Also caspase cascade activity is essential in both pathways, thus a short description of caspase proteins as well as two apoptosis pathways will be provided. The BCL-2 family will be discussed in detailed as the expression of genes of this family is the central part of this study.

1.2 The role of caspases in apoptosis

Horvitz's group for the first time discovered the role of caspases in regulation of programmed cell death during development of the nematode worm *Caenorhabditis elegans* (*7*), and because of his efforts in this field, he was awarded the 2002 Nobel Prize for studies of the genetic regulation of *C. elegans* development (*8*) . Most of the morphological changes that are observed during apoptosis are caused by a set of cysteine proteases activated specifically in apoptotic cells. These death proteases are very similar to each other in their sequence, and are part of a large protein family known as the caspases. Caspases are highly conserved through evolution, and can be found from nematodes to humans. Over a dozen caspases have been identified in mammals; about two-thirds of them have been suggested to function in apoptosis (*9*) (Table1).

Table 1. Caspases involving in apoptosis in mammals

Modified from (*6*)

Initiator Caspases	Effector Caspases
Caspase 2	Caspase 3
Caspase 8	Caspase 6
Caspase 9	Caspase 7
Caspase10	-

All known caspases possess an active-site cysteine, and cleave substrates after aspartic acid residues (*10*). A caspase's distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (*11*). Caspases have been divided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities. Caspases can be thought of as the central executioners of the apoptotic pathway because they cause most of the morphological changes that differentiate apoptotic cells from other types of dying cells. Indeed, eliminating caspase activity, either

through mutation or applying pharmacological inhibitors, will slow down or even prevent apoptosis (*12*). Thus, blocking caspases can increase cell survival.

Activation of caspases does not result in the degradation of all of the cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins, usually at one or more positions in the primary sequence and always after an aspartate residue. In most cases, caspase-mediated protein cleavage results in inactivation of the target protein, which can be either a single polypeptide chain enzymes, such as poly ADP-ribose polymerase, or a complex macromolecular network (*4*). Caspase-mediated cleavage of specific substrates explains several of the apoptotic morphological features. For example, cleavage of the nuclear lamins is required for nuclear shrinking and blebbing (*13*). However, caspases can also activate proteins, either directly, by cutting off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit. Several important caspase substrates have been identified in recent years. One of the most exciting discoveries was the elucidation of the mechanism of activation of the nuclease responsible for nucleosomal degradation. Wyllie *et. al.* first described that nuclease cuts the genomic DNA between nucleosomes to generate DNA fragments of approximately 180 base pairs. The presence of this DNA ladder has been used extensively as a marker for apoptotic cell death (*14*). Wang and Nagata showed that the DNA ladder nuclease (caspase-activated DNase, or CAD) is present in living cells prior to apoptosis as an inactive complex with an inhibitory subunit, called ICAD (*15*); caspase-3-mediated cleavage of ICAD activates CAD and causes the release and activation of the catalytic subunit (*16*).

Since caspases activity plays very important role in apoptotic process, a proper understanding of apoptosis is required to understand how caspases are activated. Similar to other proteases, caspases are synthesized as an inactive pro-enzyme. Most caspases are activated by proteolytic cleavage of the pro-enzyme between the p20 and p10 domains. Interestingly, all these cleavage sites occur at Asp-x sites- candidate caspase substrate sites- suggesting the possibility of autocatalytic activation (*17*). Indeed, the simplest way to activate a pro-caspase is to expose it to another, previously activated caspase molecule (Fig.2).

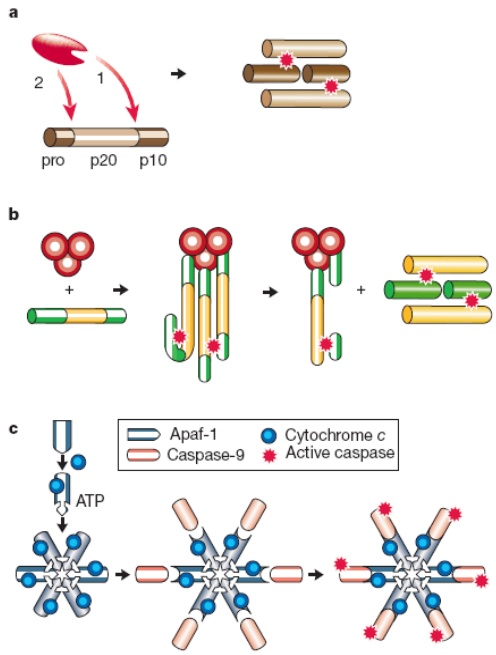


Figure 2. Mechanisms of caspases activation

Mechanisms of caspase activation include (a) proteolytic cleavage by an upstream caspase, (b) induced proximity, and (c) holoenzyme formation.

Adapted by permission from Macmillan Publishers Ltd: [Nature] (*1*), copyright (2000)

This "caspase cascade" strategy of caspase activation is used extensively by cells to activate three short prodomain caspases, caspase-3, -6 and -7. These three downstream effector caspases are considered the most active members of the caspase family, and are usually more abundant than other ones. The caspase cascade is a useful method to amplify and integrate pro-apoptotic signals inside the cell. The entire caspase activation cascade is summarized in figure 3; effector caspases are usually activated by an upstream caspase, whereas initiator caspases are activated through regulated protein–protein interactions. The actual molecular mechanisms mediating initiator caspase activation are still unclear and, most likely, much more complex that currently understood.

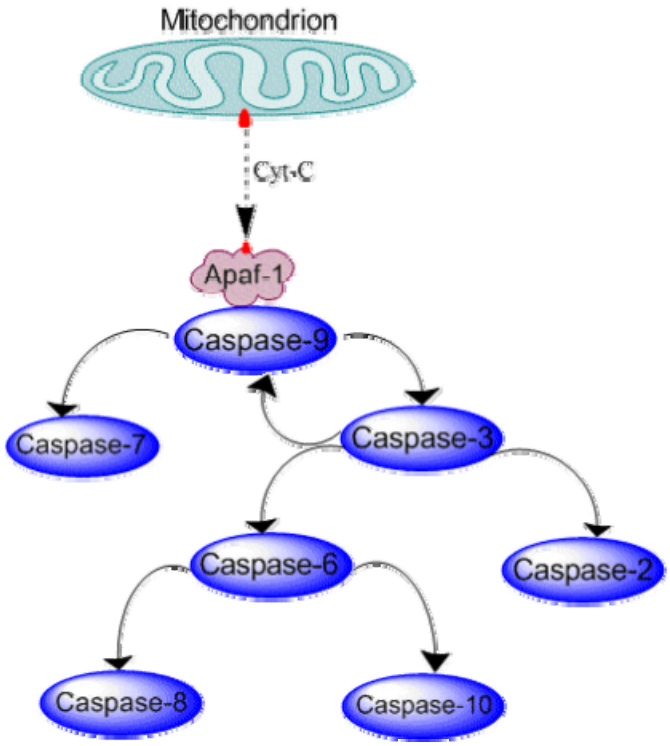


Figure 3. Order of caspase activation

Caspase cascade results in Cytochrome C release from Mitochondria into the cytosol. Modified from (*4*)

1.3 Extrinsic apoptotic pathway

The extrinsic or death receptor pathway is activated by extracellular ligands such as CD95L and tumor necrosis factor (TNF-α) that bind to death-receptor superfamily members such as CD95 and tumor necrosis factor receptor I and/or II (TNFRI, TNFRII). The CD95L /CD95 receptor and the TNFR/TNF-α are the most well-known initiators of the extrinsic pathway (*12*). Binding of these ligands to their receptors induce receptor clustering and formation of a death inducing signalling complex (Fig.4). This complex recruits pro-caspase-8 through the adaptor molecule FADD (Fas-associated death domain protein). Pro-caspase -8 activates caspase-8 which stimulates cleavage and activation of several different effector caspases. Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP (*18, 19*).

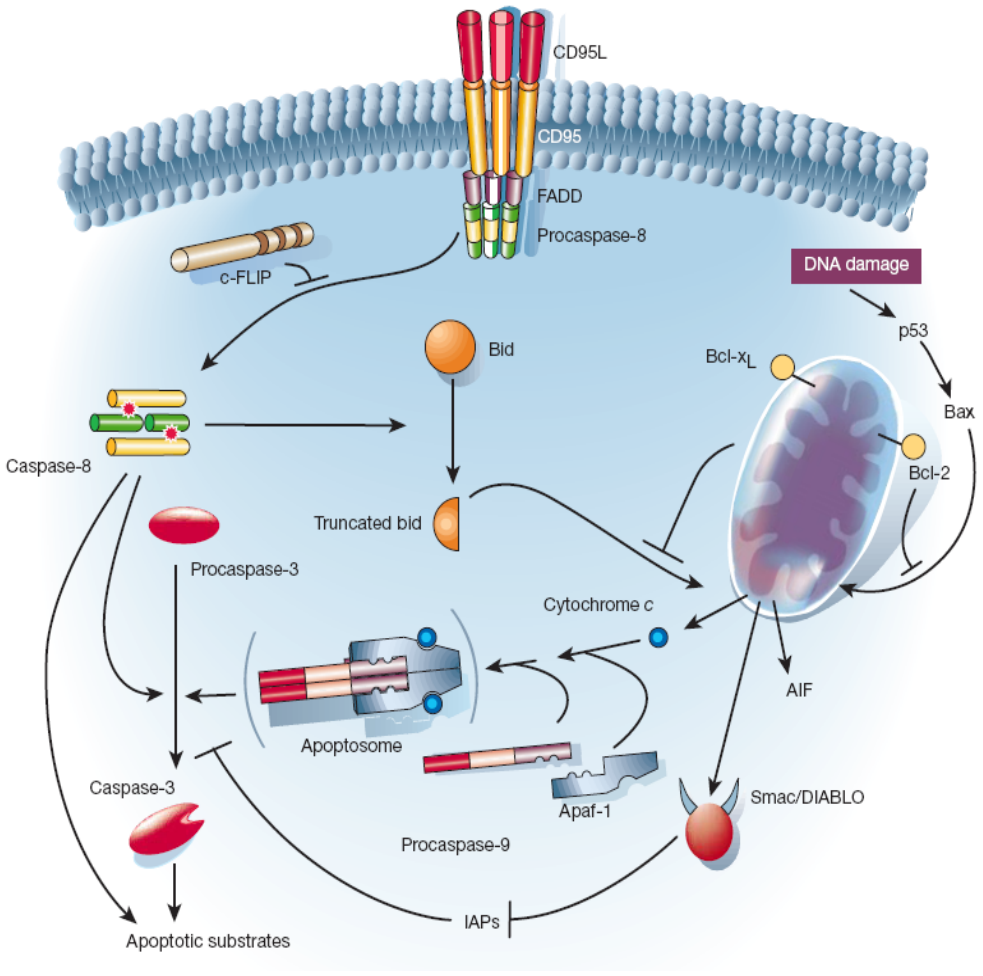


Figure 4. Two major apoptotic pathways in mammalian cells

Adapted by permission from Macmillan Publishers Ltd: [Nature] (*1*), copyright (2000)

1.4 Intrinsic apoptotic pathway

The intrinsic or mitochondrial apoptotic pathway is activated in response to either external or internal signals including DNA damage (*18*). In addition, this pathway can be activated when Cytochrome C is released from the intra-membrane space of the mitochondria into the cytosol (Fig.4). This release is under tight control by the BCL-2 family of proteins.

The Bcl-2 protein is usually attached to the intracellular membranes such as endoplasmic reticulum and mitochondria; however, some other members of this family such as Bax, Bad, and Bid proteins, can shuttle between the cytosol and organelles. The cytosolic forms of these proteins are inactive. Pro-apoptotic signals redirect these proteins to the mitochondria, where they are activated and initiate the destruction of the cell .

The Bcl-2/Bax ratio indicates the relative amounts of anti- and pro-apoptotic proteins of the BCL-2 family. This ratio decrease in response to death signals such as DNA damage (through p53) or reactive oxygen species (*19*). Members of the BCL-2 family stimulate release of Cytochrome C and other proteins from the mitochondrial intra-membrane space into the cytosol. Cytosolic Cytochrome C binds to apoptotic protease activating factor 1 (Apaf-1) which in turn stimulates binding to procaspase-9 to form a complex known as the apoptosome. When bound to the apoptosome, procaspase-9 is activated. Activated caspase-9 in the apoptosome activates the effector caspases 3, 6, and 7 by cleavage of their respective pro-caspases (*4*) (Fig.4 and 3).

The extrinsic and intrinsic apoptotic pathways meet each other at the level of caspase-3 activation. Caspase-3 activity is inhibited by the inhibitors of apoptosis proteins (IAP), whose activity in turn can be antagonized by the Smac (second mitochondria-derived activator of caspases) or DIABLO (direct IAP binding protein with low pI) proteins released from mitochondria. Downstream of caspase-3, the apoptotic programme results to the cell death.

Bid, which is a pro-apoptotic member of the BCL-2 family, provides the integration between two pathways. Activated caspase-8 mediates Bid cleavage to tBid and results in its translocation to mitochondria, where its pro-apoptotic activity promotes cytochrome C release by oligomerization to Bak and Bax proteins, other pro-apoptotic members of this family, and pore formation in mitochondrial membrane (*20*). Under most conditions, however, the death-receptor and mitochondrial pathways works independently with minimal “cross-talk” .

1.5 BCL-2 family and its role in apoptosis

The B-cell CLL/lymphoma 2 (BCL-2) family of genes and proteins present a critical intracellular checkpoint of apoptosis. The members of this family are categorized into two main groups. The first group consists of the anti-apoptotic members that share a high degree of structural and functional similarities with Bcl-2 protein, while the second one includes proteins that have fewer similarities to Bcl-2 and show pro-apoptotic activity. The second group is in turn divided into two subgroups, the Bcl-2-associated X protein (Bax)-like death factors and the BH3-only proteins (Table 2).

Table 2. The BCL-2 family of proteins in different organisms

Modified from (*4*)

Death function	Organism	Members	Structure
Anti-apoptotic	Mammals	Bcl-2,Bcl-XL, Mcl-1	BH Multi-domain
	C.elegans	CED-9	BH Multi-domain
	Xenopus	XRI, XRII	BH Multi-domain
Pro-apoptotic	Mammals	Bax, Bak, Bok	BH Multi-domain
		Bad, Bik, Bid	BH3-Only
	C.elegans	EGL-1	BH3-Only

Interestingly, this family of genes and their protein products are evolutionarily conserved and their structures have not been changed from nematodes to human. A number of viruses encode Bcl-2 homologs, including most gamma herpes viruses. Most of these viral homologs are anti-apoptotic, probably because viruses need to keep the infected cells alive for latent and persistent infection (*21*). One of the key features of the BCL-2 family proteins is that members of this family share sequence similarities in four BH (Bcl-2-homology region) domains—the BH1, 2, 3, and 4 domains—although not all of them poses all four domains (Fig.5). These domains correspond to a α-helical domain,

which mutagenesis studies have indicated are important for the various molecular functions and for protein-protein interactions (homo and hetero dimerization) among this family. Furthermore, most of the members contain a hydrophobic carboxyterminal transmembrane (TM) domain that is probably responsible for their membrane localization (*22*).

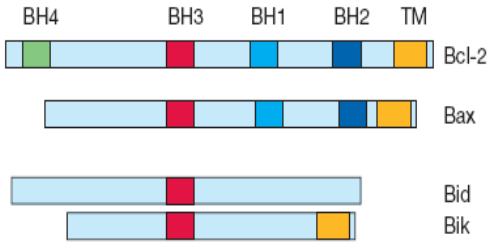


Figure 5. Schematic structure of BCL-2 family of proteins

The anti-apoptotic proteins of BCL-2 family have more co-opted by other organisms. This group usually posses three to four BH regions. Alternatively, the pro-apoptotic proteins of this family are less conserved and usually do not contain the BH4 amino terminal domain. BH3 domain is the most common feature of all family members.

Adapted by permission from Macmillan Publishers Ltd: [Nature] (*1*), copyright (2000)

The BH1 and BH2 domains are necessary for the death-repression function of the anti-apoptosis molecules, whereas the BH3 domain is required for the death-promotion function of the pro-apoptotic ones. In addition, the BH4 domain, which presents mainly in the anti-apoptotic molecules, is also important for death-inhibition functions (*22, 23*). It seems that the "BH3-only" molecules, such as Bid, Bik, and Bad, are sensors for the peripheral death signals and are able to activate the "multi-domain" executioner molecules, Bax or Bak (*24*). This process in some ways resembles the caspase cascade, in which the initiator caspases activate the effector caspases.

1.6 Protein interactions among BCL-2 family members

The BCL-2 family of proteins can interact with each other and also with several other proteins. In fact, the first pro-apoptosis BCL-2 family protein, Bax, was cloned based on

its interaction with Bcl-2. Many other BCL-2 family proteins were also cloned based on this type of interaction. The most common type of interaction is between anti-death and pro-death members, such as Bcl-2 versus Bax. This interaction can result in antagonistic action of the two types of molecules and thus could control the death program (*25*). Interestingly, not all anti-death molecules can interact with all pro-death molecules. It seems that some members of one group will preferentially bind to some members of the other group. For example, the anti-death molecule Bcl-2 binds to Bax, but not to Bak (*26*), and these molecules may only antagonize the function of those molecules to which they bind. This type of selectivity suggests that specific amino acids required for particular interactions may only exist in some but not all of the family members. In addition, it may also suggest that in certain tissues and for certain death stimuli, a specific set of BCL-2 family proteins is critically involved.

The second type of interaction occurs between two pro-death members, usually between a molecule with only one BH3 domain and a multi-domain molecule, such as Bad and Bik with Bax or Bak. Such interactions could be important for the activation of the multi-domain executioner molecules such as Bax or Bak (*26*).

The third type of interaction is “multimerization” of the same molecule. This has been observed in both anti-death molecules, such as Bcl-2 or Bcl-XL , and pro-death molecules, such as Bax and Bak (*24*). The ability of Bax or Bak to oligomerize has been considered an important factor in their function to produce or open the mitochondrial channels in purpose of releasing mitochondrial apoptotic factors such as Cytochrome C (*27*)

Overall, the relative ratio of pro-survival (Bcl-2-like) and pro-apoptotic (Bax-like and BH3 only) proteins seems to determine the cell sensitivity or resistance to the apoptotic stimuli.

Further discussion about the genes that their relative expression levels were investigated in this study is provided in detail as follow.

1.7 Death- inhibiting and promoting genes of BCL-2 family

1.7.1 Anti-apoptotic *Bcl-2*

Bcl-2 is a proto-oncogene that was identified at the chromosomal translocation breakpoint, between chromosomes 14 and 18, t (14; 18), in non-Hodgkin's follicular B-cell lymphomas. It promotes tumorogenesis by preventing cell death instead of increasing cell division rate as well as arresting cells in the Go/G1 phase of the cell cycle (*4*) . Based on this fact, *Bcl-2* was classified as a new class of oncogenes. Protein product of indicated gene is 26 kDa consisting of 239 amino acids in human and contains a single highly hydrophobic domain at its C-terminus that helps the protein to localise mainly in the mitochondrial outer membrane.

Bcl-2 gene expression was observed in a wide variety of foetal tissues, whereas it was claimed that this gene shows restricted expression in more rapidly proliferating and differentiating cells in adult tissues (*28*). *Bcl-2* gene can be up-regulated by tumour suppressor *P53* (*29*).

The main role of *Bcl-2* gene product in the process of apoptosis is based on its ability to inhibit ion conductive channels formation in mitochondrial membrane, and in most cases to prevent mitochondrial disruption and the release of Cytochrome C. Therfore, Bcl-2 protein can inhibit the following association of Cytochrome C with Apaf-1 and therefore suppress the activation of caspase-9 (*4*). Based on this scenario, two anti-apoptotic roles are suggested for Bcl-2 protein which in one of them its activity is mitochondrial dependent and in the next one Bcl-2 may play a role in the inactivation of initiator caspases, such as caspase-2, that acts upstream or independently of Cytochrome C release and mitochondrial involvement (*30*).

1.7.2 Anti-apoptotic *Bcl-X*

BCL-2L1 (BCLX, BCL-2L, BCL-X). The BCL-2-like 1 (BCL-2L1) gene maps on chromosome 20q11.21 and consists of 3 exons and 2 introns, encoding a 233 amino acid protein, localised to the outer mitochondrial membrane. This gene undergoes alternative splicing, and, up to now, three different splicing variants have been identified including

the *Bcl-XL*, Bcl-XS, and Bcl-Xγ. The large transcript *Bcl-XL* is pro-survival and mainly expresses in long-lived cells and localises in the mitochondrial membranes (*31*).

Bcl-XL protein shows high similarities with Bcl-2 and contains the BH1 to BH4 conserved domains as well as a hydrophobic region at its C-terminus. According to different crystallography reports, the BH1, BH2 and BH3 motifs of Bcl-XL are very close to each other, thus, forming a "hydrophobic pocket" through that the BH3 region of pro-apoptotic molecules interact with this protein (*4*). For example, the presence of an anti-apoptotic molecule, such as Bcl-2 or Bcl-XL, can inhibit the activation of the pro-apoptotic Bax. Therefore, Bcl-XL, like the Bcl-2 protein, regulates programmed cell death by interaction and blocking the death-promoter proteins. Minn *et. al.*, in 1997, suggested that *Bcl-XL* may regulate survival by regulating the permeability of the intracellular membranes, preventing the removal of the Cytochrome C in cytosol, and taking care of the membrane integrity (*32*). On the other hand, it has been reported that Bcl-XL can bind to Apaf-1, like the Bcl-2 function, and then form a complex that prevents the activation of caspase-9 (*33*).

The *Bcl-X*L gene is transcriptionally reactive (*34*). Its expression is up-regulated by irradiation (*35*), and insulin-like growth factor-1 (IGF-1) (*6, 36, 37*).

1.7.3 Pro-apoptotic *Bax*

Apte *et. al.* in 1995 applying somatic cell hybrid and in situ hybridization showed that the human *Bax* gene is located on chromosome 19q13.3–q13.4 (*36*).This gene consists of 6 exons and 5 introns that encode a 21 kDa protein contains the three conserved regions BH1, BH2 and BH3, very similar to Bcl-2 structure, which provide the susceptibility to form hetero-dimers with Bcl-2 and imply its death promoting function (*35*). The formation of hetero-dimers between Bax and the other members of the BCL-2 family play important role in the regulation of cell death scenario (*4*).

It seems that the tumour suppressor *P53* can transcriptionally regulate *Bax* expression. Actually, in vitro experiments have shown that *P53* up-regulates the *Bax* gene, while *in vivo* studies using *P53* -/- mice have indicated the opposite results (*37*) .

Bax is the first death-promoting member of the BCL-2 family to be identified, and it was co-immunoprecipitated with Bcl-2 (*25*). Different studies have demonstrated that in

healthy cells Bax protein is localized in cytosol in monomeric conformation, after exposure to a death signal, this protein translocates to the mitochondria and homodimerizes with another Bax or heterodimerizes with other pro-apoptotic members such as Bak protein (*38*).

The presence of anti-apoptotic molecules such as Bcl-2 and Bcl-XL, can inhibit the activation of Bax following a death signal (*39*). However, the death promoting function of Bax is not associated with its ability to interact with these molecules. Bax mediates apoptosis through a mitochondrial-mediated pathway that can be either caspase-associated or not, with the caspase-dependent mitochondrial pathway being based on the release of cytochrome C from the mitochondrial membranes (*4, 35*).

Bax is an important gene for the control of cell death. Cells that over express this gene show enhanced apoptosis, whereas *Bax*-null cells are resistant to apoptosis (*25*). *Bax* expression has also been associated with tumour development (*35*).

1.7.4 Pro-apoptotic *Bak*

Bcl-2 homologous antagonist/killer 1 *(Bak1)* has been cloned as a Bcl-2-related gene, which consists of 6 exons and maps to chromosome 6p21.3 on the human genome,(*40*), encoding a 211-amino acid protein with a relative molecular weight of 23400 (*41*). *Bak* gene product is very similar to Bax protein structurally and functionally. This protein contains the conserved domains BH1 to BH3 and enhances apoptotic cell death (*25*). The widespread tissue distribution of Bak messenger RNA suggests that cell-death-inducing activity is broadly distributed, and tissue-specific modulation of apoptosis is controlled primarily by regulation of molecules that inhibit apoptosis (*6*). Furthermore, Leu *et. al.* in 2004 demonstrated that *Bak* is regulated at the transcriptional level, with over expression of *P53* (*42*). The anti-apoptotic Bcl-XL interacts with Bak and inhibits its activity (*43*).

1.7.5 Pro-apoptotic *Bad*

Bad or *Bcl-XL/Bcl-2*-associated death promoter is a death-promoting member of the BCL-2 family that was initially detected because of its ability to interact with the anti-apoptotic proteins Bcl-XL and Bcl-2. This gene maps to chromosome 11q13.1 and its

protein product is about 168 amino acids. The interesting fact about Bad protein is this protein neither hetero-dimerizes with other members of the family, such as pro-apoptotic Bax, nor homodimerizes. Bad forms an inactivating dimer with Bcl-XL, but doesn't have any effect on the anti-apoptotic activity of Bcl-2 because this protein only has the BH3 domain, which is essential for its heterodimerization with the other family members, and slight similarity with Bcl-2 (*6, 44*). Kaipia *et. al.* in 1997 indicated that the Bad protein shows a wide tissue distribution with higher expression levels in lung, ovary, uterus and brain (*45*). It seems that the active form of Bad, which bound to Bcl-2 and Bcl-XL in the mitochondria, is the dephosphorylated form, while some factors such as IGF-1 inactivates this protein through phosphorilation (*6, 25*). It has been suggested that *Bad* mediates apoptosis through a caspase-dependent pathway, since the cell death could be inhibited in granulosa cells, which already had shown apoptosis because of over expression of *Bad* mRNA, using a caspase inhibitor (*45*). Therefore, the association of *Bad/Bcl-2/Bcl-XL* results in the release of Cytochrome C, which in turn, activates the proteolytic caspase-mediated cascade (*25*).

1.7.6 Pro-apoptotic *Bik*

The *Bik* gene maps on chromosome 22q13.3 and translates to a 19kb protein with 5 exons and 4 introns (*46*).It has been demonstrated applying Northern blot analysis that *Bik* gene expression is elevated in heart and skeletal muscle (*46*) and restricted in kidney, pancreas, lung, liver, prostate and testis (*6*).

Although the protein heterodimerizes with anti-apoptotic molecules, it has been found that this interaction is not sufficient to cause death. It has been reported that the heterodimerization with death-inhibiting proteins such as Bcl-2 and Bcl-XL is not enough for the death-promoting effect of the complex. Mutation analysis by deletion of the BH3 domain showed that the pro-apoptotic activity is present even in the absence of the heterodimerizationion (*6*). This fact shows that Bik protein mediates apoptosis through a mechanism which is not dependent on its dimerization. *Bik* also may be rapidly turned over during apoptosis (*47*).

1.8 BCL-2 family of genes in colon cancer

1.8.1 Apoptosis in Colon

Cancerous cells have some characteristics that are not present in their normal counterparts. Normal cells, for example, are subject to internal and external inhibitory signals, which are lost during carcinogenesis. A decreased rate of cell death is as important as increased proliferation in determining the probability of a cell changing to a pre-cancerous state. When normal cells differentiate they divide slowly, but cancerous cells use the "dedifferentiation trick" to increase their growth rate. Also, they loosen their contact with other cells or with the extracellular matrix to escape from growth inhibition.

In colonic epithelium keeping a constant number of cells is critical. The high incidence of colon cancer makes understanding of the balance between cell proliferation and apoptosis even more important. Histologically, colon tissue is made up of four distinct layers of mucosa and muscle, the most inner layer of the mucosa is the colonic epithelium and contains invaginations known as crypts (*48*). Cell proliferation, migration, differentiation and apoptosis are organized along the crypt axis. Normal colonic crypts are composed of four distinct layers which include stem cells at the base, a proliferation and a differentiation zone in the lower third of the crypt, a migration zone in the upper two-thirds, and the surface epithelium where old cells will be eliminated by apoptosis, respectively (*49*).

Newly produced colonic epithelial cells migrate from the base of the crypt to the surface epithelium within 6–7 days. The normal construction of the crypt is maintained by a balance between cell proliferation in the bottom and apoptosis at the top of the crypt and surface epithelium (*50*). In 1998 Lengauer and Vogelstein showed that increased proliferation and decreased apoptosis were the main features present in neoplastic colon. In addition, malignant neoplasms are the consequence of activation and/or suppression of regulatory genes that control cell proliferation and apoptosis (*51*). The accumulation of mutations in these genes is common and produces the malignant nature of neoplasm. In normal colonic crypts, cycling cells may undergo mutations during their migration along the crypt but these genetically altered cells will be eliminated if the apoptotic machinery

functions properly at the surface epithelium (*50*). However, some mutations may cause resistance to apoptosis thus leading to tumorogenesis.

Since colorectal cancer remains the third most frequent fatal cancer in the western world and most sporadic cases of colon cancers go through progressive stage (*52*), keeping the balance between cell proliferation and apoptosis and also assessing the expression levels of the regulatory genes and proteins is very important.

1.8.2 The role of BCL-2 family and mitochondrial apoptotic pathway in colon cancer progression

Apoptosis inhibition plays critical role in tumorogenesis. Cell death regulating genes and proteins seem to be good candidates for molecular cancer therapy and among them, the members of BCL-2 family are prominent targets. Deregulation of BCL-2 family members has been tightly linked to tumorogenesis (*53*). All the anti-apoptotic members seem to function as oncogenes, and pro-apoptotic ones can act as tumour suppressors. This is supported by a comparative genomic hybridization (CGH) database search for copy number gains and/or deletions of chromosomal loci of members of the BCL-2 family. This indicated specific roles for these genes and their pro- and anti-apoptotic products during the pathogenesis of tumours (*6*). Every pro- and anti-apoptotic members of the BCL-2 family plays specific roles in cancer development. For example, *Bcl-2* gene was found to be over-expressed in some types of human tumors such as colon, gastric, lung, and breast cancer (*6*). In some cases, such as lung and breast cancers, *Bcl-2* up-regulation can be used as a prognostic marker (*54, 55*), while in other types, it is still under investigation. Huang *et. al.* in 2002 showed that in some cells, such as lymphoma cells, the phosphorylation/ dephosphorylation of the Bcl-2 protein plays an important role in apoptosis, and therefore, can be involved in carcinogenesis (*56*). Researchers applying immunohistochemical methods showed that the Bcl-XL protein is widely expressed in different types of cancer cells such as colon, breast and ovarian cancers and over-expression of the *Bcl-XL* gene can promote tumor formation in mice. Also *Bcl-XL* expression is induced by a wide range of survival signals (*57*).In opposite, in another study it was shown that the *Bcl-XL* gene can be down-regulated in colon cancer, which can be applied as a prognostic marker of this malignancy (*58*). Since the Bad protein is

hyperphosphorylated in colorectal and other types of cancers as well as different malignant cell lines, heterodimerization of Bad and Bcl-2 would decreased in this situation (*59*), which consequently reduce the pro-apoptotic activity of this protein. This result was confirmed four years later, when it was shown that the phosphorylated form of Bad protein cannot prevent tissues from excessive growth (*6*) since the phosphorylated form of this protein in inactive.

In healthy cells, Bax contributes to tissue homeostasis through the induction of apoptosis to remove of excessive and damaged cells. However, in cancer cells the concentration of this protein is reduced, which in some cases is accompanied by mutation in the *P53* tumor suppressor gene (*25*). Indeed, currently, the potent tumour-suppressing activity of this protein is used for therapeutic purposes (*60*). Based on immunohistochemical studies it has been shown that *Bak* expression is altered in cancerous cells. In colorectal carcinomas, for instance, the increased levels of Bak and Bcl-2 heterodimerization contribute to tumour growth (*59*). In normal gastrointestinal epithelial tissues, expression of *Bak* gene is up-regulated during differentiation, which is essential for normal cell turnover, whereas in gastric tumours *Bak* level is down-regulated compared to the normal mucosa . Down regulation of the *Bak* gene could perhaps be explained by missense mutations in the *Bak* gene (*61*). It was explained previously that in many of colorectal and breast tumours the *Bik* gene expression is lost due to up-stream silencing mutations, therefore the absence of this gene is implicated in development of these cancer types (*6*). Moreover, in 2002 it was suggested that since certain chemotherapeutic agents induce pro-apoptotic Bik protein over-expression and sensitize tumour cells to apoptosis this protein could be applied as a potential therapeutic target for human cancer (*62*).

Colon tumors are developed from a serious of somatic mutations subsequent to an initiation event such as DNA methylation. Experimental carcinogens that are known to induce gastrointestinal tumors in rodents such as 1,2-dimethylhydrazin and its derivative azoxymethane (AOM) are mostly methylating agents. These chemical agents were documented not only as gastrointestinal carcinogens, also as causes of other cancers for human after a lifetime exposure (*63*). In fact, in some disorders such as colon cancer and

hepatic steatosis, DNA damage removal either through repair systems or apoptosis is critical in these disorders. Previously, it was described that after carcinogen injection and during the initial phases of tumorogenesis, there is an immediate apoptotic response to DNA damage in colon epithelium (*64*). One year later, the same research group indicated that the regulation of apoptosis-related protein Bcl-2, Bcl-XL, and Bax was altered in AOM induced colonic neoplastic tissue in Sprague Dawley rats, while no significant difference was found between the expression of these proteins in the non-neoplastic mucosa of the AOM treated rats and normal mucosa of saline treated control group (*65*). These results show that apoptotic gene and/or protein expressions in early initiation phase would be different from late phase of AOM induced colon tumorogenesis. However, these results could be different in obese and lean Zucker rats.

1.9 BCL-2 family of genes in liver disease

Non-alcoholic fatty liver disease or NAFLD is a disease of our generation as it was mostly unrecognized before 1980 and was not taken seriously until the past few years. In fact, this disease was recognised after the industrial revolution, when individual lifestyles were changed. Since NAFLD has roots in lifestyles and is related to obesity, physical activity and diabetes, it is now accepted as a metabolic disorder. This section will discuss liver tissue and its function as well as hepatic steatosis and its consequent problems, including the role of apoptosis in the progression of liver disease.

1.9.1 Liver tissue and obesity related disease

Liver represents about 2% of the total body weight. Liver function is comparable to the function of stomach, intestine, pancreas and kidney together. In fact, all digested foods are taken up by the intestine and then by the liver, which then stores the energy derived from the oxidation of the nutrients. Therefore, liver is the power source of the body. Liver is responsible for important functions such as energy storage, metabolism, production and secretion of the serum proteins, as well as processing of absorbed nutrients, bile acid synthesis and bile formation. Although the liver is made up of several cell populations, the most abundant cell type by mass and by number is the hepatocyte. Hepatocytes play role in maintenance of glucose, amino acid, ammonia and bicarbonate homeostasis in body, as well as the storage and processing of signal molecules (*66*). Therefore, keeping the well-organized physiological function of hepatocytes is very critical. Steotasis is described as fatty liver and characterized by fat droplet accumulation in hepatocytes (*67*).

In 1980, Ludwig and his colleagues defined the term of nonalcoholic steatohepatitis (NASH) as a form of liver injury that was very similar to alcoholic hepatitis but was noticed in obese, diabetic patients, who denied alcohol use . Nonalcoholic steatohepatitis or NASH is a kind of metabolic liver disease in which fatty change or steatosis is associated with inflammation, hepatocyte injury and/or hepatic fibrosis. This problem, in fact, is the advanced stage of non-alcoholic fatty liver disease or NAFLD which is a liver syndrome that describes a range of liver abnormalities from simple triglyceride

accumulation in hepatocytes (hepatic steatosis) to some more dangerous pathologic cases such as steatohepatitis, progressive liver fibrosis and even hepatocellular carcinoma (*68*). There are multiple factors that have been associated with NAFLD and NASH such as obesity, diabetes type 2, insulin resistance and hyperinsulenemia, hyperlipidemia, lifestyle and lack of physical activity (*69*). Among all of them, obesity and insulin resistance are the most important promoting factors. The majority of patients with NAFLD do not show any symptom of their disease initially, but later hepatomegaly is the first common physical sign (*70*). Correction of insulin resistance by diet and increased physical activity is a logical approach to prevent early NASH.

According to previous studies, hepatic steatosis is a pre-requisite for events that lead to liver injury; however, the mechanisms by which hepatic steatosis progress to NASH/NAFLD is poorly understood (*71*). A growing body of evidence suggests that increased programmed cell death in hepatocytes plays an important, if not critical, role in liver disease progression (*72*).

1.9.2 Apoptosis in Hepatocytes

More frequent hepatocyte death has been increasingly noticed as an intermediate step between liver injury and fibrosis (*73*). Studies using a genetic model of NAFLD, obese (ob/ob) mice, that are deficient in leptin, have shown a significantly increased expression of some apoptotic mediators such as TNF-α and cytochrome P4502E1 (CYP2E1) mRNA, and oxidative stress (*74*). Also, it was explained previously that obese patients with NASH have enhanced expression of TNF-α mRNA, whereas obese patients without NASH do not (*75*), which shows that obese individuals are probably more prone to extrinsic apoptosis pathway. *In vitr*o exposure of human hepatocytes to long chain saturated free fatty acids results in a "dose dependent" induction of apoptosis (*76*). As previously described by Canbay *et. al.*, death receptor-mediated apoptosis is particularly prominent in the liver (*77*). In 2004, Takehara and collaborators showed that disruption of *Bcl-XL* expression in hepatocytes can lead to continuous apoptosis of these cells and result in liver disorders (*72*). Another study has demonstrated that hepatocyte apoptosis and Fas protein expression are up regulated in patients with NASH disorder (*78*). *In vitro*

studies using a liver cancer cell line have revealed that free fatty acids also promote up-regulation of the death receptor (*79*) as well as redistribution of pro apoptotic Bax protein from cytosol to lysosome that in turn induces mitochondrial membrane channel formation (*80*), releasing Cytochrome C in to the cytosol and activation of caspases-3 and -7, and thus cell death machinery.

1.10 Animal model of obesity

The fa mutation on Fatty (Fa) gene, which is located on chromosome 5 in rats, for the first time was discovered in 1961 by Zucker and results in non-functional leptin receptor (*81*). Homozygote animals (fa/fa) become noticeably obese by three to five weeks of age compare to their heterozygote (Fa/fa) or homozygote (Fa/Fa) lean counterparts. The Zucker (fa/fa) rats are the best-known and most widely used rat model of obesity that has been extensively used for investigations of obesity-associated diseases. The obesity in these animals is inherited as a Mendelian recessive trait. Obese animals suffer from hyper-lipemia, hypercholesterolemia (*82*), as well as hyper-insulinemia (*83*). Leptin hormone is produced by adipose tissues and is responsible for regulating fat metabolism and control body weight by signalling the hypothalamus to suppress appetite (Guyton *et. al.*, 2000). Non-functional leptin receptor in (fa/fa) Zucker rats results in uncontrolled appetite and become morbidly obese.

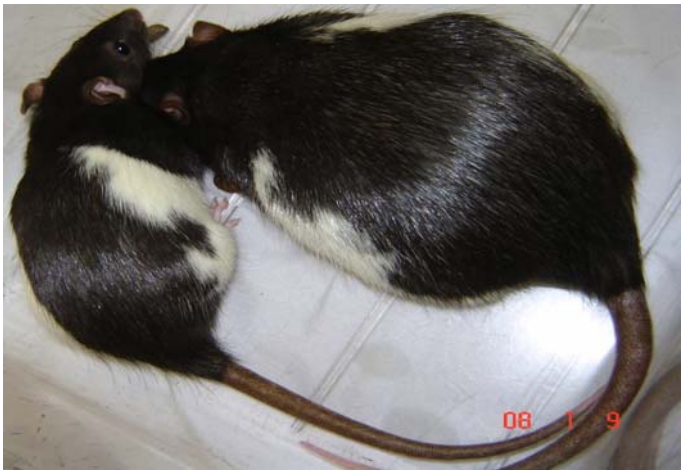


Figure 6. Lean and obese Zucker rats

Seven-week-old Lean (Fa/?) Zucker rats with functional leptin receptor (left) and Obese (fa/fa) Zucker rats with non-functional leptin receptor (right).

1.11 Objectives of current study

The objective of this study was to investigate the differences between pro- and anti-apoptotic gene expression levels in liver and colon tissues of obese and lean individuals to investigate whether or not the BCL-2 family of genes play role in the obesity effected progression of hepatic steatosis and colon cancer.

The specific goals of this study were,

1) To compare pro- and anti-apoptotic gene expression levels in hepatic tissue and colonic mucosa in obese rats versus lean rats,

2) To compare the reaction of obese and lean animals to azoxymethane (AOM) induced apoptosis,

3) To determine if the effect of obesity on colon cancer and hepatic steatosis could be predicted by an analysis of apoptotic gene expressions during the first 24 hours post administration of the carcinogen AOM.

Chapter 2 Materials and Methods

2.1 Animal care

Seven week old female Obese (fa/fa) rats (n=30) and their lean (Fa/?) counterparts (n=30) were purchased from Charles River Laboratory (Wilmington, MA, USA) and housed in suspended wire cages approximately 10 cm above a sawdust bedding tray with a 12h light/12h dark cycle, in the animal housing facility, Department of Biology, University of Waterloo. Temperature and relative humidity were controlled at 22˚C and 55% respectively. All animals were handled in the above conditions for 10 days with free access to standard laboratory rodent chow and drinking water until initiation of the experiment. All animals were cared according to the guidelines of the Canadian Council on Animal Care and the protocol was approved by the University of Waterloo Animal Care committee.

Both groups of animals were randomly weighed and the average weight of lean and obese animals was 111.07±9.0g and 182.4±8.4g, respectively, values with 95% confidence interval.

2.2 Body Weights, AOM Injection and Termination

At eight weeks of age, obese and lean rats were randomly weighed again and the average weights of lean and obese animals were 148±8.5g and 268±11.4g, respectively.

Ten days after accommodation, all animals, except the control group, were injected with colon specific carcinogen Azoxymethane (AOM) at the doses of 10mg/Kg body weight. Obese animals and their lean counterparts were then randomly divided into four groups. Group 1 (n=6) was a non-injected control group, Group 2 (n=8) was terminated 3h after injection of AOM, Group 3 (n=8) was terminated 9h after injection of AOM, and Group 4 (n=8) were terminated 24h after injection of AOM (Figure 2). After the injection, at three different time intervals, all animals were euthanized by CO2 and terminated.

Figure 7. Schematic representation of the experimental design

Liver and distal colon mucosa was removed rapidly, gross anatomies of indicated tissues were observed and any pathologic abnormalities were recorded. Samples were then transferred to RNAlater™ stabilizer solution (Sigma, Canada), snap-frozen in dry ice and stored in -80°C. Weights of liver as well as other tissues such as kidney, spleen, and heart were recorded and these samples were also frozen for future research.

2.3 Preparation of Total RNA from Zucker Rat Liver and Colon Mucosa

Analysis of RNA levels is a method for gene expression studies. Isolation of total RNA of full-length is the first and may be the most critical step in analyzing RNA levels. Total RNA from eukaryotic RNA contains primarily ribosomal RNA (rRNA) and transfer RNA (rRNA) and in a much lower amount messenger RNA (mRNA). In this study, total RNA was extracted from liver and colon mucosa samples using a GE Healthcare RNAspin mini RNA isolation kit (http://www.gehealthcare.com/lifescience), according to the manufacturer's direction.

15mg of liver and 25mg of colon tissue (scraped mucosa) were sliced over dry ice (to keep tissues frozen) and submerged in to 350µl of ice-cold RA1 (buffer) and 3.5µl β-mercaptoethanol, and then homogenized on ice using PT2100 Polytron homogenizer. The mixture was vortexed vigorously and transferred into RNAspin Mini filter units held in 2 ml collection tubes and centrifuged at 13,200 rpm for 1 min in order to reduce the viscosity and clear the lysate. An equal volume of 70 % cold ethanol was added to the homogenized lysate and mixed via pipetting to adjust the RNA binding conditions, followed by filtration using the RNAspin mini column held in 2 ml collection tubes and

centrifuged at 10,000 rpm for 30 sec. The flow through was discarded and 350 µl of membrane desalting buffer was added to the RNeasy column, followed by a 1min 13,200 rpm centrifugation in order to desalt the filter. The DNase reaction mixture was prepared in a sterile microcentrifuge tube, for each isolation 10ul reconstituted DNaseI and 90µl DNase reaction buffer was added exactly to the centre of the silica membrane of column and incubated for 15min at room temperature. After the incubation time, DNase enzyme was inactivated by 200ul RA2 buffer and centrifuged at 13200rpm for 1min. The RNeasy column was transferred to a new 2 ml collection tube and washed by 600ul RA3 buffer containing 96-100% Ethanol and centrifuged for 1min at 13200rpm. The supernatant was discarded and another 250 µl Buffer RA3 was added and centrifuged for 2 min at 13,200 rpm. The RNeasy column was again transferred to a new 2 ml collection tube and centrifuged for 1min at 13200rpm. The RNeasy column was then transferred to a new 1.5 ml RNase, DNase, proteinase free microcentrifuge tubes. RNA was eluted by two 40µl RNase free water, followed by centrifugation at 13,200 rpm for 1 min each. RNA was stored at -80˚C. All the centrifuge steps were performed at 4˚C.

2.4 RNA Quantification

The quantity of extracted RNA samples was measured using a NanoDrop ND-1000 UV is Spectrophotometer (NanoDrop Technologies). RNA concentration and purity was determined by measuring the ratio of the UV absorbance at 260nm and 280nm. Individual samples were measured in triplicate and the average was taken. The ratio of OD260nm/ OD280nm lower that 2 was considered as protein free RNA samples.

2.5 cDNA Synthesis

cDNA was synthesized from 1ug and 500ng of the total RNA from liver and colon mucosa, respectively, using the BIO-RAD iScript cDNA synthesis kit (Bio-Rad Laboratories, Canada) following the manufacturers direction applying 2ul oligo(dT) primer and Nuclease free water to the total volume of 15ul. Then, samples were incubated for 5min at 65˚C followed by snap chilling on ice for about 1min. 4ul of 5X

reaction mixture and 1ul of reverse transcriptase were added followed by 80min incubation at 42˚C in order to cDNA synthesises reaction, 5min at 85˚C to inactivate the enzyme and then store the cDNA product at -20˚C . All the above steps were performed on ice and a nuclease free bench.

2.6 Primer Design

Primers were designed based on the genomic and mRNA sequences retrieved from gene sequence databases such as National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The location of every intron and exon was determined within the gene sequences based on the mRNA sequence in order to design the primers at exon-exon junctions to avoid the false positive results arising from amplification of possible contaminating genomic DNA. Primers were checked by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that they did not have any non-specific binding sites on either the same gene or similar sequence sites in other species.

2.7 Gradient PCR

Gradient PCR was performed using GoTaq Green Master Mix (Promega, Madison, US) in order to set up the optimum annealing temperature for each separate gene. A 25µl PCR reaction was set up as follows: 12.5ul of 2X GoTaq Green Master Mix, which contains GoTaq® DNA Polymerase that is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2; 400nM of 10uM forward and reverse primers, 200ng cDNA template, and appropriate volume of nuclease free water was added to a 0.2ml nuclease free PCR tubes and centrifuged for 10sec. The thermal cycler was adjusted as follows: initial denaturation step at 95°C for 2min, subsequent denaturation step at 95°C for 30s, optimization of the annealing conditions by performing the gradient reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature in increments of 3°C to the annealing temperature for 30sec, followed by 72°C for 25sec for

template extension and a final extension of 5 minutes at 72°C, following a 4°C incubation for 10min. All the PCR reaction preparation steps were performed on ice.

2.8 Agarose Gel Electrophoresis

The GoTaq® Reaction Buffer contains yellow and blue dyes, the blue dye migrates at the same rate as 3–5kb DNA fragments, and the yellow dye migrates at a rate faster than primers (<50bp), in a 1% agarose gel.

The PCR product was separated on 2% agarose gel containing 0.1% ethidium bromide (EtBr) and the DNA bands were visualized with a UV transilluminator containing an EtBr filter. The image system was a Fluorochem 8000 imager, which used Alpha Innotech (San Leandro, CA, USA) visible imaging software. The primer sequences and appropriate annealing temperatures are shown in the following Table.

Table 3. List of genes and designed primers

Official name	Accession number	Function	Forward Primer	Reverse Primer
Bax	NC_005100	Bcl-2-related gene; involved in the regulation of apoptotic cell death	5'CTTTTTGTTACAGGGTTTCATCCAG3'	5'CTCTGCAGCTCCATATTATTGTCC3'
Bcl-2	NC_005112	an anti-apoptotic protein; involved in inhibiting cell death in many different cell types	5′ GCTGGGATGCCTTTGTGGAA 3′	5′ CTCACTTGTGGCCCAGGTA 3′
P53		multifunctional	5′ GGAGAATATTTCACCCTTAAGATCC 3′	5′ GAGTGAGCCCTGCTGTCTCCT 3′
Bak1	NC_005119	BCL-2-antagonist/killer 1	5′ GTCCCTAGAACCCAACAGTGTCTT 3′	5′ CTTAAATAGGCTGGAGGCGAT 3′
Bik	NC_005106	may be involved in differentiation of Oligodendroglial lineage cells	5'TGGGATTGCTATGCACAGACTTG3'	5' TCAGGTGACACCCAGGCGC3'
Bad	NC_005100	may be involved in induction of programmed cell death	5'CCAGCAGCCCAGAGTATGTTCC3'	5'CATAGTCCCAGCGCCTCCA3'
Bcl-2l1	NC_005102	Also known as Bclx; Bcl-2l; bcl-X; *Bcl-XL*	5′ CTATCTTGGCTTTGGATCCTGGA 3′	5′ CTCCCGGTTGCTCTGAGACAT 3′
Beta Actin	J00691	Housekeeping gene	5′ CGTGCGTGACATTAAAGAGAA 3′	5′ CGCTCATTGCCGATAGTGAT 3′
18srRNA		Housekeeping gene	5′ CCTACTTGGATAACTGTGGTA 3′	5′ GTTATCTAGAGTCACCAAAGC 3′

2.9 Real-Time PCR set up

Real time detection of the PCR product was performed with the inclusion of the PCR reaction mixture with SYBR Green fluorescent dye. BioRad Real-Time thermal cycler machine equipped with fluorescence detector cameras was used to monitor the accumulation of the amplified product in each cycle.

The same amount of tissue (15mg of liver and 25mg of colon tissue) was used for RNA extraction. Then the same amount of RNA (1ug and 500ng of the total RNA from liver and colon mucosa, respectively) was used to synthesize the cDNA. Finally, the expression of a reference gene, *β-actin* or *18srRNA* housekeeping genes, was chosen to normalize and control for any difference in the cDNA input amount and pipeting errors.

2.9.1 Standard curve creation

The accuracy of PCR reaction for each individual gene was verified using standard curve analysis and Ct values. For this purpose, 10-fold cDNA serial dilutions was prepared and 2ul of each dilution was mixed with 30µl of 2X iQ SYBR Green Supermix, which contains 100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase 50 units/ml, 6 mM MgCl2, SYBR Green I, 20 nM fluoresein, and stabilizers; 6ul of cDNA sample. Appropriate final concentrations of cDNA template as well as particular primers were measured by performing a PCR reaction for each individual gene using primer concentration gradient variable between 300 to 100nM. Results of these reactions were summarized in table 4.

Table 4. Appropriate concentrations of designed primers

Primer Name	Primer Concentration
Bax	150nM
Bcl-2	200nM
P53	100nM
Bak	200nM
Bik	100nM
Bad	100nM
Bcl-XL	200nM
Beta Actin	100nM
18srRNA	150nM

Following primer addition, appropriate volume of nuclease free water was added to a 0.2ml nuclease free microcentrifuge tubes to the final volume of 60µl, centrifuged for 10sec and aliquot as 20ul in each well of an ice-cold 96well PCR plates. PCR plates were then centrifuged for 1min at 4˚C. All the above steps were performed on ice and a nuclease free bench. The iQ5 iCycler PCR system from BOI-RAD Real-Time system was set up at 95°C for 3min for the initial denaturation step, at 95°C for 30s for the subsequent denaturation step, the annealing temperature that was variable for each individual gene (refer to table 3) for 30s, followed by 72°C for 20s for template extension and a final extension step for 3 minutes at 72°C. The PCR efficiency was calculated applying the following formula

$$E = 10^{-(1/\text{slope})}$$

$$\% \text{ Efficiency} = 100\ (E-1)$$

PCR reactions with efficiency between 95% and 105% were accepted; otherwise the PCR reaction for that specific gene was performed again with different primer concentration and annealing temperature.

2.9.2 Melt curve analysis

The melt curve analysis was performed for each separate gene to prove the accuracy of primers and show the possible genomic DNA contamination. In order to perform the Real-Time RT-PCR reactions, all the cDNA samples were diluted 10 times using 1X sterile, nuclease free TE buffer (10mM Tris with pH 8.0 and 0.1mM EDTA) from Roche Applied Science (http://www.roche-applied-science.com). PCR reaction was carried out using the BIO-RAD iQ SYBR Green 2step RT-PCR kit (Bio-Rad Laboratories, Canada) in 60µl volume consisting of 30µl of 2X iQ SYBR Green Supermix, 6µl of diluted cDNA sample, certain concentration of up- and down-stream primers, which was variable for each individual gene as described in previous section, and appropriate volume of nuclease free water was added to a 0.2ml nuclease free microcentrifuge tubes, centrifuged for 10sec and dispensed as 20µl in each well of a ice-cold 96well PCR plates. PCR plates were then centrifuged for 1min at 4˚C. All the above steps were performed on ice and a nuclease free bench. cDNA samples were amplified performing the following conditions: 95˚C for 3min to activate the enzyme followed by 40 cycles of 95˚C for10s to denature, appropriate annealing temperature for each individual gene for 30s, and 72˚C for 20s to extend the template samples, with a final extension step for 3min at 72˚C; and melt curve creation step which was adjusted for each gene separately started at 5˚C above the annealing temperature and ended at 95˚C with 1˚C increment/cycle and hold 10s/cycle.

2.10 Real-Time PCR Results Analysis

The two most commonly used methods to analyze data from real-time, quantitative PCR experiments are absolute quantification and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The 2-ΔΔCt method, which for the first time in 2001 was proposed by Livak, is a reliable method to analyze the relative changes in gene expression from real-time quantitative PCR experiments. In the present study, this analysis method was applied for the first objective to find out the differential gene expression levels in non-injected obese animals relative to

their lean counterparts, and for the second objective, injected obese and lean animals relative to the non-injected control groups. PCR reaction was performed for each individual sample in triplicate and the average of Ct values was taken.

$$\Delta Ct\ (Control) = Ct\ Target\ gene - Ct\ Reference\ gene$$

$$\Delta Ct\ (Test) = Ct\ Target\ gene - Ct\ Reference\ gene$$

$$\Delta\Delta Ct = \Delta Ct\ (Test) - \Delta Ct\ (Control)$$

$$Relative\ fold\ change = 2^{-\Delta\Delta Ct}$$

2.11 Statistical Analysis

The average of the relative expression level of mRNA of each individual tested group was taken and plotted a bar graph using Microsoft Excel. The standard deviations and the error bars were also calculated and added on the graph. Statistical analysis of the data was carried out using SPSS statistical software (SPSS Inc., Chicago, IL, USA). A comparison between lean and obese groups was performed and differences were determined using independent samples t-test, at a significance level of $P < 0.05$. Also, a comparison between the overall effect of DNA damage induction in obese and lean tested groups at different time points post AOM administration was performed using the one-way ANOVA test in conjugation with Tukey's post hoc test, and the overall comparison between pro- and anti-apoptotic gene expression levels in liver and colon tissues within 24 hours post AOM injection was carried out using two-way ANOVA test followed by a Tukey's post hoc test and for all tests, $p \leq 0.05$ was considered significant.

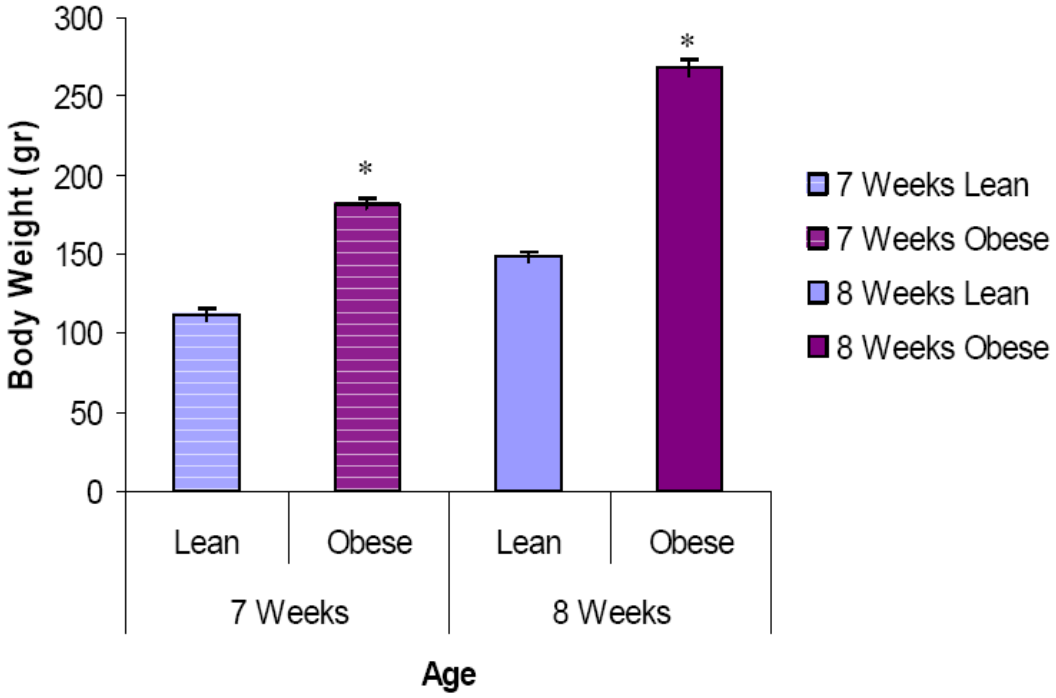
Chapter 3 Results

3.1 Animal weights

As described in the previous chapter, three days after arrival and acclimatization, a subset of seven-week-old obese and lean Zucker rats were weighed and the average weight of these animals was 111.07±9.0 g for lean and 182.4±8.4 g for obese group. One week later the same animals were weighed again and the average weights of lean and obese animals were 148±8.5 g and 268±11.4 g, respectively. It was previously described that obese Zucker rats usually start to show overweight compare to lean animals at three to five weeks of age. This demonstrates that the body mass of obese rats used in this study was significantly higher than their lean counterparts with a 99% confidence interval.

Figure 8. Lean and obese animals' body mass at the age of seven and eight weeks

Bar graphs indicate the average of animal body weight in each group ± S.E.M. at the age of seven and eight weeks; the asterisk (*) on the obese groups indicates that they have a significantly higher body weight than their lean counterparts ($P<0.01$; Lean group N=11; Obese group N=12).


300
250
200
150
100
50
0
Body Weight (gr)
*
*
Lean
Obese
Lean
Obese
7 Weeks
8 Weeks
Age
7 Weeks Lean
7 Weeks Obese
8 Weeks Lean
8 Weeks Obese

3.2 Apoptotic related gene expressions in obese and lean Zucker rats

In this project, the effect of obesity as a metabolic disorder on gene expression levels of the pro- and anti-apoptotic members of the BCL-2 family of genes, good candidates for apoptotic index measurement, was investigated. Liver and colon were examined since obese individuals are more prone to colon cancer and liver disorder progression. Therefore, applying a paired t-test analysis the effect of obesity on apoptotic gene expression levels was assessed in obese versus lean individuals to find out the significant differences in obese compare to lean groups.

3.2.1 The effect of obesity on pro- and anti-apoptotic gene expression levels in colon tissue

In this part of the study, it was hypothesized that without any external insult, obesity does affect the pro- and anti-apoptotic gene expression levels in colon tissue.

As shown in Fig.9, the anti-apoptotic *Bcl-2* gene expression level is significantly lower in obese animals compare to their lean counterparts. The pro-apoptotic *Bak, Bad* and *Bax* genes are significantly up-regulated in obese animals. It seems that other genes followed the same expression level in both groups.

3.2.2 The effect of obesity on pro- and anti-apoptotic gene expression levels in liver tissue

In this part of the study, it was hypothesized that pro- and anti-apoptotic gene expression levels in liver tissue were affected by obesity genotype without external signals. Fig.10 shows that the apoptotic regulator gene *P53* and its downstream genes, anti-apoptotic *Bcl-XL*, and pro-apoptotic *Bax* and *Bak* are significantly up-regulated in obese individuals. Pro-apoptotic *Bad* and *Bik*, and anti-apoptotic *Bcl-2* gene expression levels seem to show the same trend in both groups and were not significantly changed.

Figure 9. Colonic pro- and anti-apoptotic gene expression profiles in non-injected Zucker rats

Gene expression levels in distal colon mucosa in obese rats were assessed using quantitative Real-Time RT-PCR relative to their lean counterparts (base line) and normalized using *B-actin* housekeeping gene as an internal control. Bar graphs represent mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in obese colonic mucosa. Tissues were collected from 12 rats and expressions were measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.

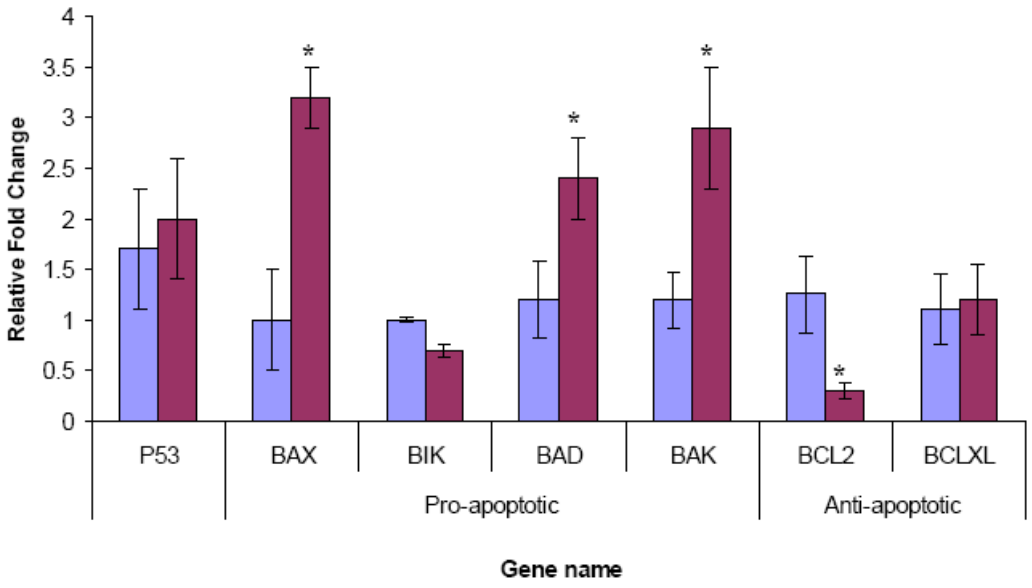
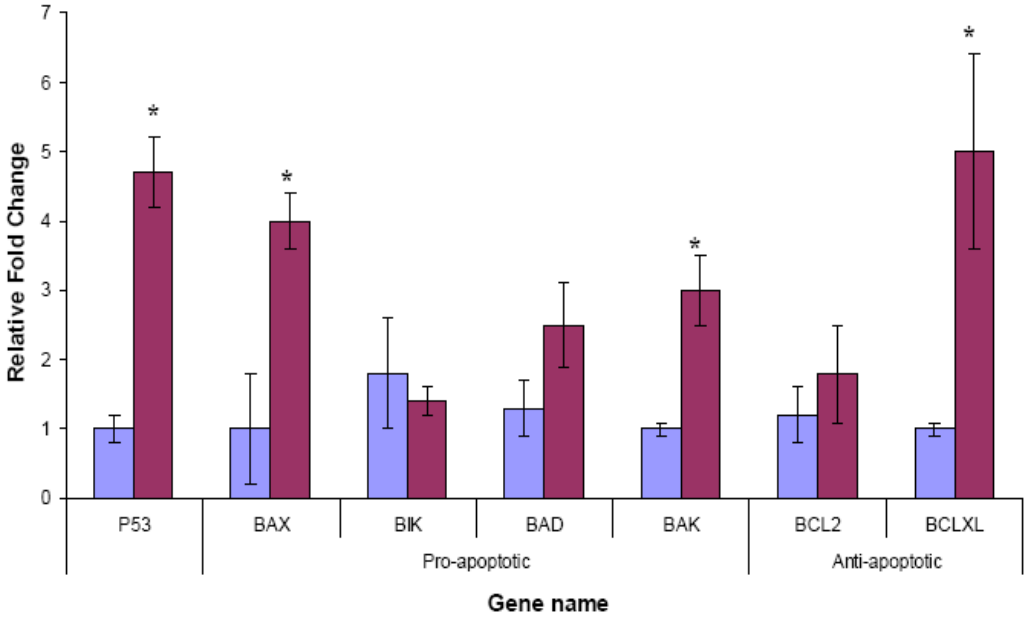

Relative Fold Change
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

Figure 10. Hepatic Pro- and anti-apoptotic gene expression profiles of non-injected Zucker rats

Gene expression in the liver of obese rats was assessed using quantitative Real-Time RT-PCR and the final results were described relative to control lean animals (base line) and normalized using the *18srRNA* housekeeping gene as an internal control. Bar graphs represent relative mean ±S.E.M of the fold change of individual pro- and anti apoptotic genes in obese hepatocytes. Tissues were collected from 12 rats and expressions were measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue and red bars represent apoptosis gene expression levels in lean and obese animals, respectively.


Relative Fold Change
7
6
5
4
3
2
1
0
*
*
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

3.3 Differential apoptotic related gene expressions in Zucker rats after AOM administration

pro- and anti-apoptotic gene expression levels was measured in AOM injected obese and lean Zucker rats relative to the non-injected lean animals to follow the effect of obesity on apoptosis gene expression levels at three different time points- at three, nine, and twenty-four hours- post AOM injection. This part of current study was performed to more finely determine the possible changes in gene expression levels in obese animals compare to their lean counterparts after apoptotic signal induction. It was hoped that the results would elucidate the possible role of apoptosis on progression of colon cancer and liver dysfunction in obese individuals. All the results were statistically analyzed by paired t-test to find out the significant differences of gene expression levels between obese and lean individuals at each particular time point.

3.3.1 BCL-2 family gene expression levels in colonic mucosa of Zucker rats after AOM injection

All Zucker rats, except the control group, were injected with carcinogen AOM as an internal apoptotic signal inducer. Following this, eight animals in each group were terminated at three different sampling times in order to determine the effect of initial stages of DNA damage on anti- and pro-apoptotic gene expression levels of the BCL-2 family as well as the possible synergistic effect of obesity on expression of these genes.

As shown in Fig.11, at three hours after AOM injection, the apoptotic regulator gene *P53* and its downstream pro-apoptotic gene expression levels are significantly different between obese and lean animals. *P53, Bax,* and *Bad* genes show higher expression levels in obese individuals, while *Bik* and *Bak* genes are expressed at lower levels than those seen in their lean counterparts. Although pro-apoptotic gene expressions are altered, anti-apoptotic gene expression levels have not been changed at three hour post treatment in obese individuals compare to their lean counterparts.

Results in Fig.12 indicate that apoptotic regulator gene *P53* and its downstream pro-apoptotic *Bad* gene expression level is significantly lower in obese than in lean individuals at nine hours post AOM injection; however, pro-apoptotic *Bax* gene expression level is significantly higher in obese group at this time point. Two pro-apoptotic *Bik* and *Bak* genes as well as the two anti-apoptotic genes, *Bcl-2* and *Bcl-XL*, are expressed at the same level in both groups.

Twenty-four hours after carcinogen administration, three pro-apoptotic genes- *Bax, Bad* and *Bik* - show significantly higher expression levels in obese individuals, while the other genes are expressed at similar level obese and lean animals (see Fig.13).

Figure 11. Colonic pro- and anti-apoptotic gene expression profiles in Zucker rats at three hours post AOM injection

Three hours after carcinogen administration, the gene expression levels in distal colon mucosa in both lean and obese rats were assessed using quantitative Real-Time RT-PCR relative to the control lean group (N=6) and normalized to the housekeeping gene *B-actin* as an internal control. Bar graphs represent mean ±S.E.M of relative fold change of individual pro- and anti apoptotic genes in lean and obese colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$).Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.

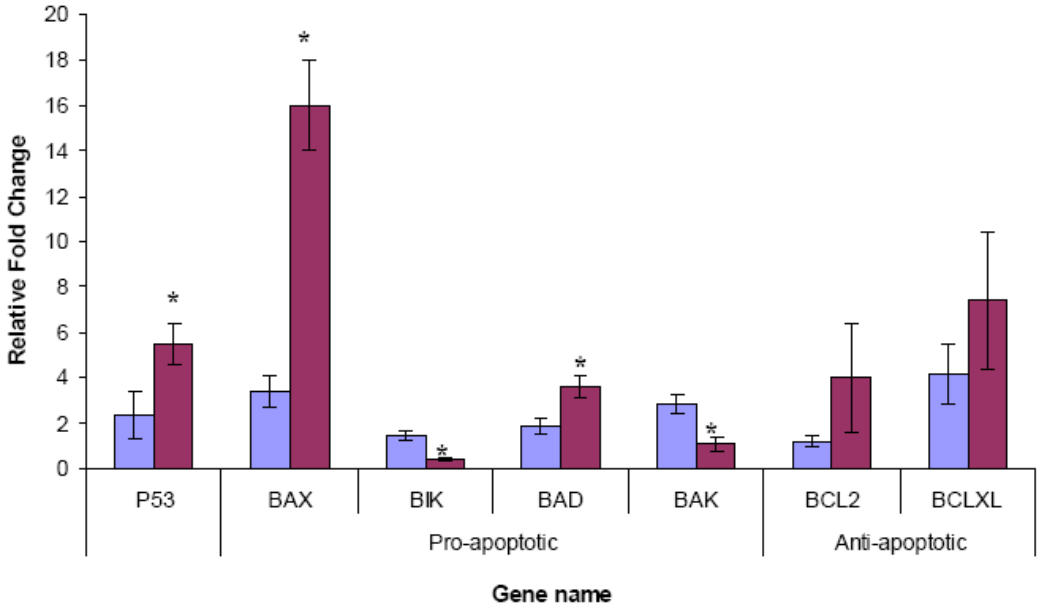

Relative Fold Change
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

Figure 12. Colonic pro- and anti-apoptotic gene expression profiles in Zucker rats at nine hours post AOM injection

Nine hours after carcinogen administration, the gene expression levels in distal colon mucosa in both lean and obese rats were assessed using quantitative Real-Time RT-PCR relative to the control lean group (N=6) and normalized to the housekeeping gene *B-actin* as an internal control. Bar graphs represent the mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in obese and lean colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.

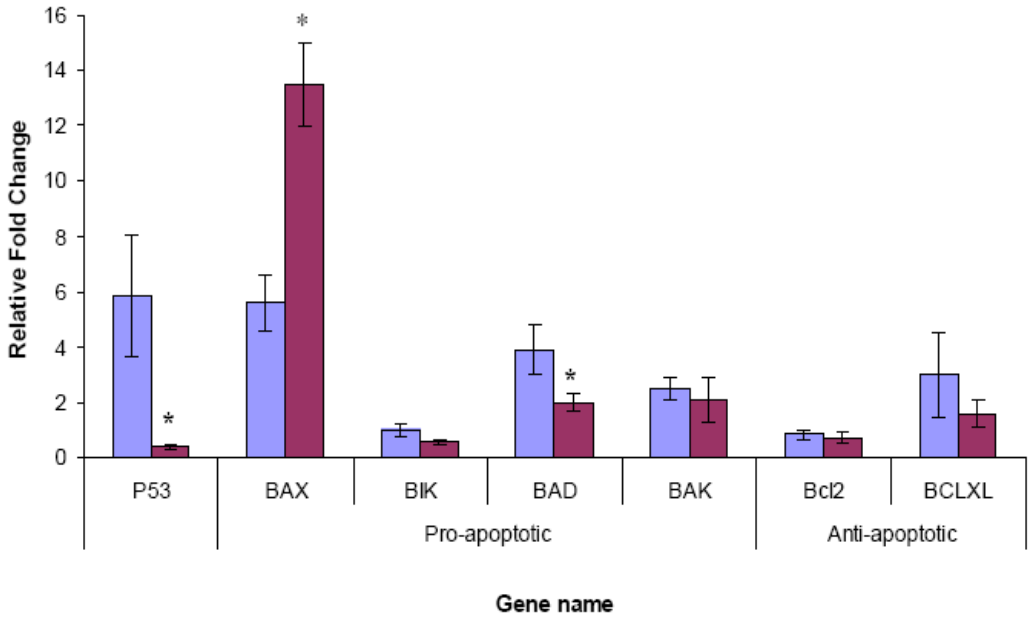
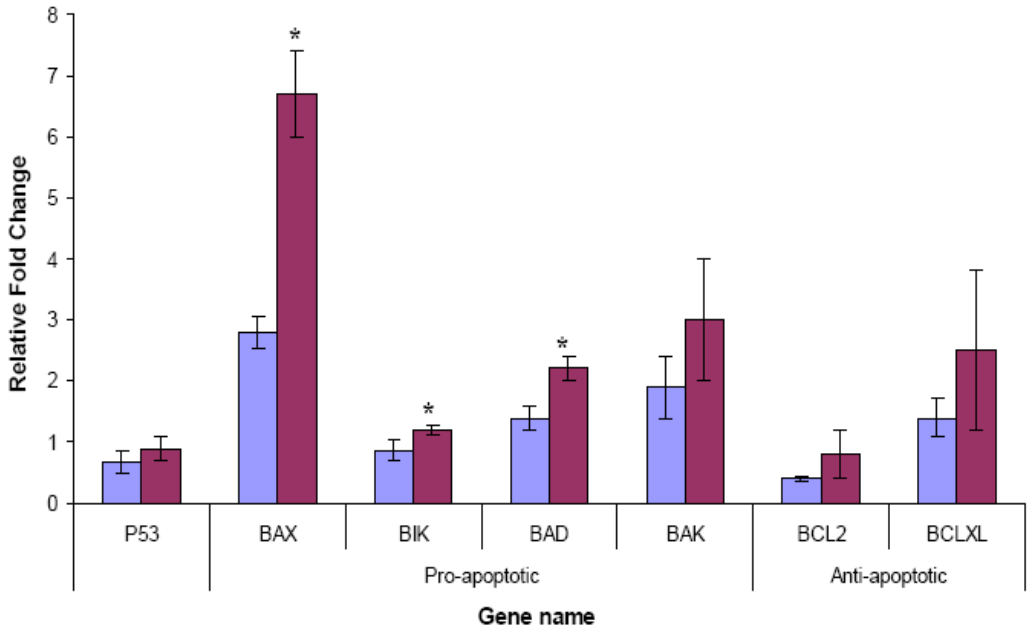

16
14
12
10
8
6
4
2
0
Relative Fold Change
*
*
*
P53
BAX
BIK
BAD
BAK
Bcl2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

Figure 13. Colonic pro- and anti-apoptotic gene expression profiles in Zucker rats at 24 hours post injection

Twenty-four hours after carcinogen administration, the gene expression levels in distal colon mucosa of lean and obese Zucker rats were assessed using quantitative Real-Time RT-PCR relative to the control lean group (N=6) and normalized to the housekeeping gene *B-actin* as an internal control. Bar graphs represent the mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in lean and obese colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.


Relative Fold Change
8
7
6
5
4
3
2
1
0
*
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

3.3.2 BCL-2 family gene expression levels in liver tissue of Zucker rats after AOM injection

All obese and lean Zucker rats, except 12 rats, which constituted the control group, were injected with carcinogen AOM as an internal apoptotic signal inducer. Following this, eight animals in each group were terminated at three different sampling times. Following termination, very tiny part of the liver tissue was subjected to further study. Gene expression analysis was performed on extracted mRNA in order to determine the effect of initial stages of DNA damage on anti- and pro-apoptotic genes of the BCL-2 family in this tissue. The possible effect of obesity on expression of these genes and its possible effect on liver disease progression were investigated as well. It was hypothesized that AOM treatment would affect the expression level of indicated genes and this effect would differ between obese and lean individuals.

As indicated in Fig.14, apoptotic regulatory gene *P53* and its downstream gene pro-apoptotic *Bax* is significantly up-regulated in obese animals at three hours after carcinogen administration. Other pro- and anti-apoptotic genes show the same expression level in both groups of animals. Based on the results on Fig.15, at nine hours post AOM injection, pro-apoptotic *Bax* expression level is higher in obese individuals compare to their lean counterparts. The other genes do not show significantly different expression levels in these two individual groups. Although it seems that *Bad* expression is lower in obese individuals, statistically this difference is not significant because the P value is 0.056. Fig.16 demonstrates the expression level of pro- and anti-apoptotic genes in obese and lean Zucker rats at twenty-four hours after AOM treatment. At this particular time, both pro- and anti-apoptotic genes, *Bax, Bak, Bad* and *Bcl-2*, show significantly higher expression levels in obese individuals, tumor suppressor gene *P53* show lower expression level in obese animals and the other genes seems to have the same expression level in both groups.

Figure 14. Hepatic pro- and anti-apoptotic gene expression profiles in Zucker rats at three hours post AOM injection

Three hours after carcinogen administration, the gene expression levels in liver tissue of Zucker rats (N= 16) were assessed using quantitative Real-Time RT-PCR relative to the non-injected lean animals (N=6) and normalized to the housekeeping gene *18srRNA* as an internal control. Bar graphs represent the mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in lean and obese colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.

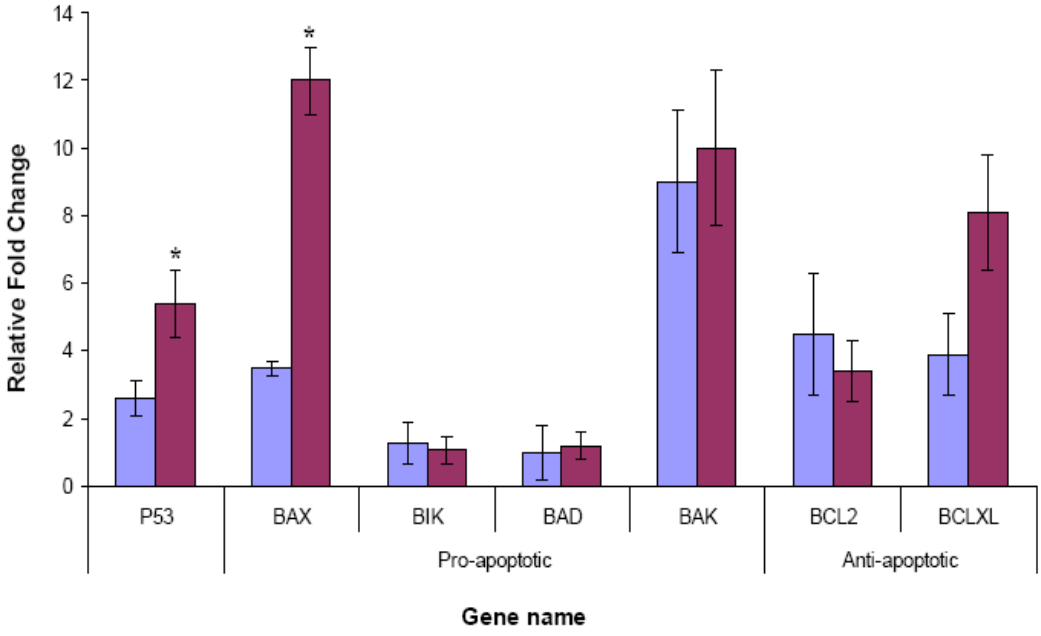

Relative Fold Change
14
12
10
8
6
4
2
0
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

Figure 15.Hepatic pro- and anti-apoptotic gene expression profiles in Zucker rats at nine hours post AOM injection

Nine hours after carcinogen administration, the gene expression levels in liver tissue of both obese and lean Zucker rats (N= 16) were assessed using quantitative Real-Time RT-PCR relative to the non-injected lean animals (N=6) and normalized to the housekeeping gene *18srRNA* as an internal control. Bar graphs represent the mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in lean and obese colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.

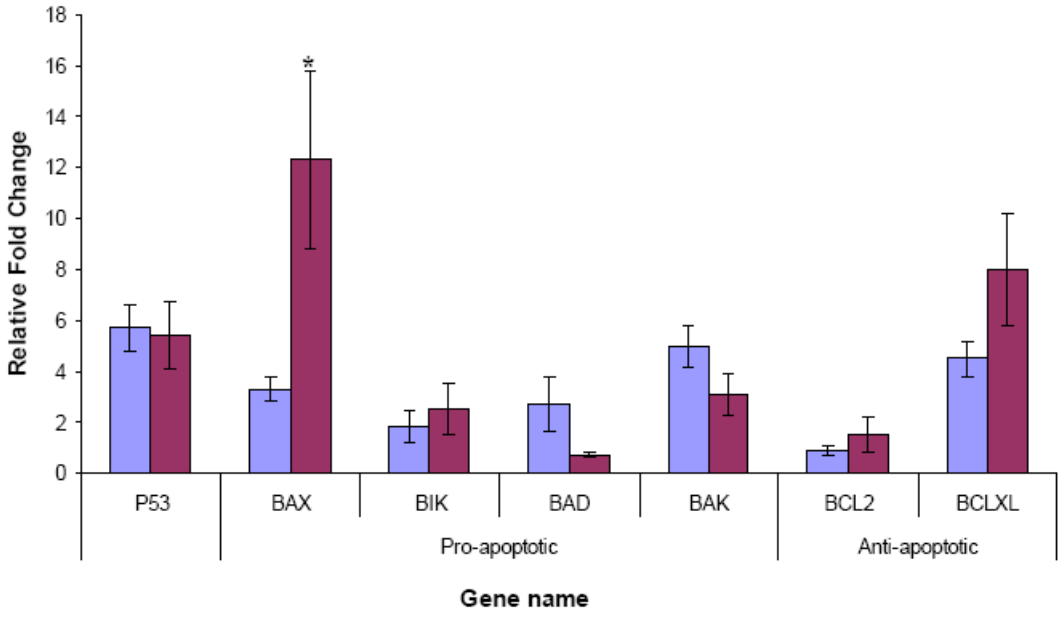
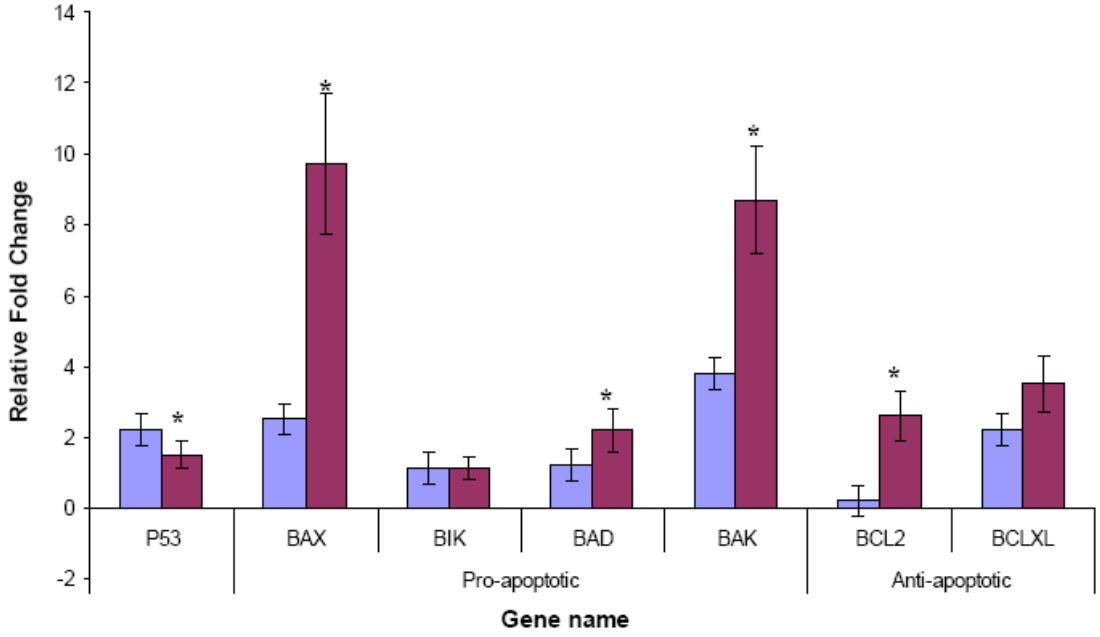

Relative Fold Change
18
16
14
12
10
8
6
4
2
0
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

Figure 16. Hepatic pro- and anti-apoptotic gene expression profiles in Zucker rats at 24 hours post AOM injection

Twenty-four hours after carcinogen administration, the gene expression levels in liver tissue of both obese and lean Zucker rats (N= 16) were assessed using quantitative Real-Time RT-PCR relative to the non-injected lean animals (N=6) and normalized to the housekeeping gene *18srRNA* as an internal control. Bar graphs represent the mean ±S.E.M of the relative fold change of individual pro- and anti apoptotic genes in lean and obese colonic mucosa. Tissues were collected from 16 rats and expression was measured in triplicate. The asterisk (*) indicates a significant difference from the lean group ($P<0.05$). Blue bars represent gene expression in lean animals, while red bars represent expression in obese animals.


14
12
10
8
6
4
2
0
-2
Relative Fold Change
*
*
*
*
*
P53
BAX
BIK
BAD
BAK
BCL2
BCLXL
Pro-apoptotic
Anti-apoptotic
Gene name

3.4 Colonic BCL-2 gene expression patterns in Zucker rats after AOM injection

In this part of the study, it was hypothesized that obesity would affects the BCL-2 family of gene expression patterns in colon tissue and obese individuals show different apoptotic involving gene expression patterns within 24 hours post AOM injection from their lean counterparts. In order to accomplish this, the overall effect and overall differences of the gene expression patterns in these individual groups were examined statistically.

As it is shown in Fig.17-A, obese and lean animals do not show the same *Bcl-2* gene expression pattern in colon tissue. The one way ANOVA test shows that overall, within 24 hours after AOM injection, obese animals show significantly different *Bcl-2* gene expression pattern compare to their lean counterparts (P= 0.034). This achievement gives support to the results of the Tukey HSD test. This complementary test revealed that there are significant differences at three and twenty four hours post AOM injection (P=0.02) compare to non-injected animals at time zero. As seen in Fig.17-B, obese and lean Zucker rats show a significantly different *Bcl-XL* gene expression pattern in colon tissue within twenty-four hours after AOM injection (P=0.024). The Tukey HSD test revealed that there are significant differences at three and twenty-four hours after carcinogen injection and in both groups anti-apoptotic *Bcl-XL* gene expression at three hours post treatment is significantly higher than what it is at 24 hours (P=0.04).

As seen in Fig.17-C, one way ANOVA test showed that *Bax* overall gene expression pattern is significantly different in obese and lean Zucker rats within 24 hours after AOM injection (P=0.01). In obese individuals, *Bax* gene expression is significantly up-regulated after AOM administration and reaches its peak at three hours post injection, and the results of following Tukey post hoc test revealed that at this time point its expression level is significantly higher than what it is at time 0 and 24. In lean animals, *Bax* expression reaches its peak at nine hours after treatment that is significantly higher than time 0 and 24. In both groups, *Bax* expression is significantly down-regulated after nine hours post injection, and at twenty-four hours after AOM administration its expression level is significantly lower than what it is at nine hours (P=0.00).

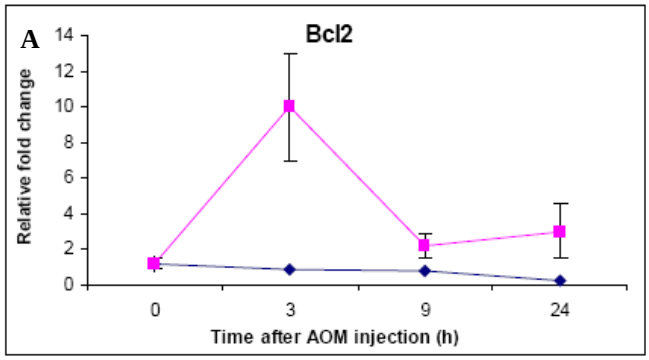
The overall one way ANOVA revealed that *Bad* gene expression pattern, within twenty-four hours after AOM injection, in obese animals is significantly different from their lean counterparts (P=0.002) with a peak at three hours post injection, while its gene expression level reaches the peak in lean animals at nine hours post injection. However, the Tukey HSD test does not show any significant difference (Fig.17-D). Moreover, *Bik* gene expression pattern is significantly different in obese animals from their lean counterparts and shows significantly lower level in obese individuals (P=0.000) (Fig17-E). Statistical testing indicated that in obese individuals, the *Bik* gene is down-regulated over 24 hours after AOM injection, but in lean animals is up-regulated over the time. However, in both groups, its expression level at twenty-four hours post injection is significantly different from time 0 (P=0.006). Within the same time intervals, obese and lean Zucker rats showed an opposite *Bak* expression pattern and based on the overall ANOVA results, with significantly lower expression in obese animals (P=0.001). In lean individuals, *Bak* is up-regulated after AOM administration with the highest level at three hours post injection, while in obese rats it is down-regulated with the lowest expression level at particular time point (Fig.16-F). However, the Tukey HSD test revealed that there are no significant differences among time points 0, 3, 9, and 24 hours post injection.

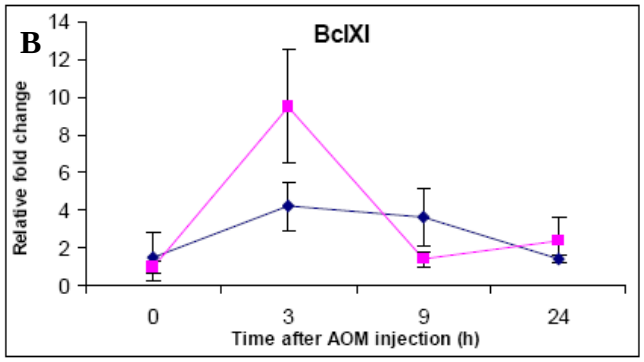
It seems that dynamically anti-apoptotic genes follow the same expression patterns in obese individuals but different patterns in their lean counterparts (Fig.17-A and B). Furthermore, the pro-apoptotic gene expression patterns are different from one gene to another and each particular gene shows dynamically specific pattern in obese and lean individuals (Fig.17-C, D, E and F). However, *Bad* gene expression pattern in obese animals is very similar to anti-apoptotic genes (Fig.17-D and A,B).

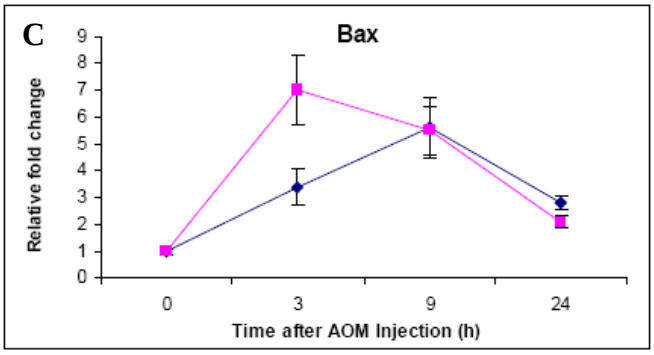
Fig.18 shows the *P53* gene expression pattern following AOM administration. As the ANOVA results indicated, there is an overall significant difference between *P53* gene expression patterns in obese and lean individuals within 24 hours post AOM injection (P=0.001). In obese individuals, *P53* expression peak is at three hours post injection while in their lean counterparts is at nine hour. Further analysis applying the Tukey post hoc tests revealed that in either obese or lean individuals, at three hours after injection *P53* expression is significantly higher than what it is at time zero and twenty four hours (P=0.008).

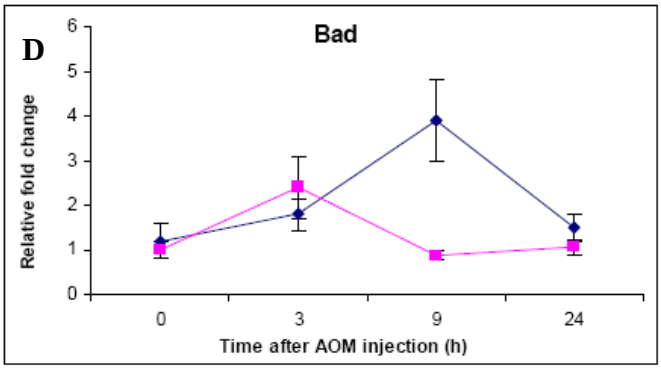
Figure 17. Anti- and pro-apoptotic gene expression patterns in colon epithelium of Zucker rats after AOM injection

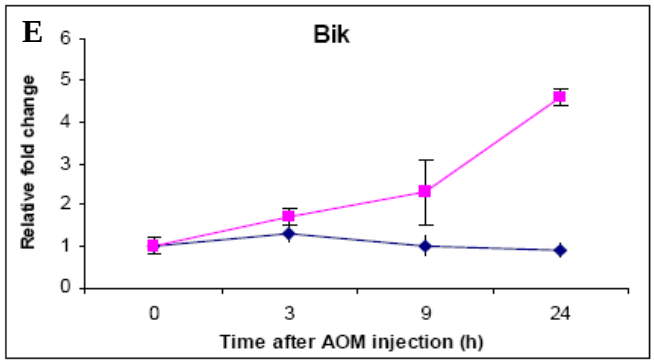
Obese and Lean Zucker rats were injected with carcinogen AOM and terminated at three different time intervals. Time 0 indicates the control group that were not injected. The anti-apoptotic and pro-apoptotic gene expressions were assessed using quantitative Real-Time RT-PCR relative to the non-injected animals in each individual group and normalized to the housekeeping gene *B-actin* as an internal control. The anti-apoptotic *Bcl-2* and *Bcl-XL* results were presented in panel A and B, respectively, and pro-apoptotic *Bax, Bad, Bik*, and *Bak* gene expression patterns were presented in sections C, D, E, and F, respectively. Line graphs represent the mean ±S.E.M of the relative fold change of individual genes at four different time points in obese and lean colonic mucosa. Tissues were collected from 60 rats, 30 obese and 30 lean, and each individual gene expression was measured in triplicate. The asterisk (*) indicates a significant difference over the time ($P<0.05$). Blue graphs represent gene expression pattern in lean animals, while red graphs represent gene expression pattern in obese animals.


A
Bcl2
Relative fold change
14
12
10
8
6
4
2
0
0
3
9
24
Time after AOM injection (h)


B
BclXl
Relative fold change
14
12
10
8
6
4
2
0
0
3
9
24
Time after AOM injection (h)


C
Bax
Relative fold change
9
8
7
6
5
4
3
2
1
0
0
3
9
24
Time after AOM Injection (h)


D
Bad
Relative fold change
6
5
4
3
2
1
0
0
3
9
24
Time after AOM injection (h)


E
Bik
Relative fold change
6
5
4
3
2
1
0
0
3
9
24
Time after AOM injection (h)

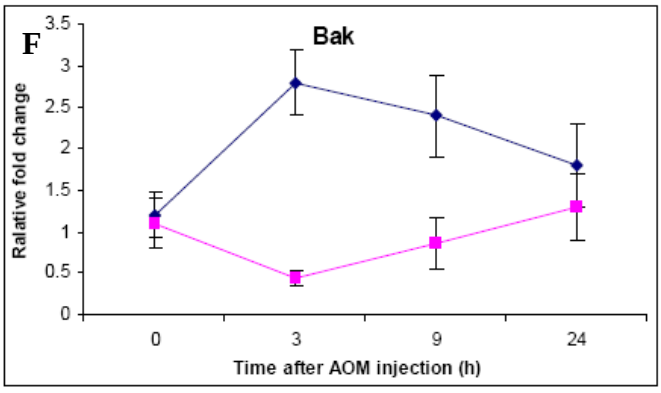
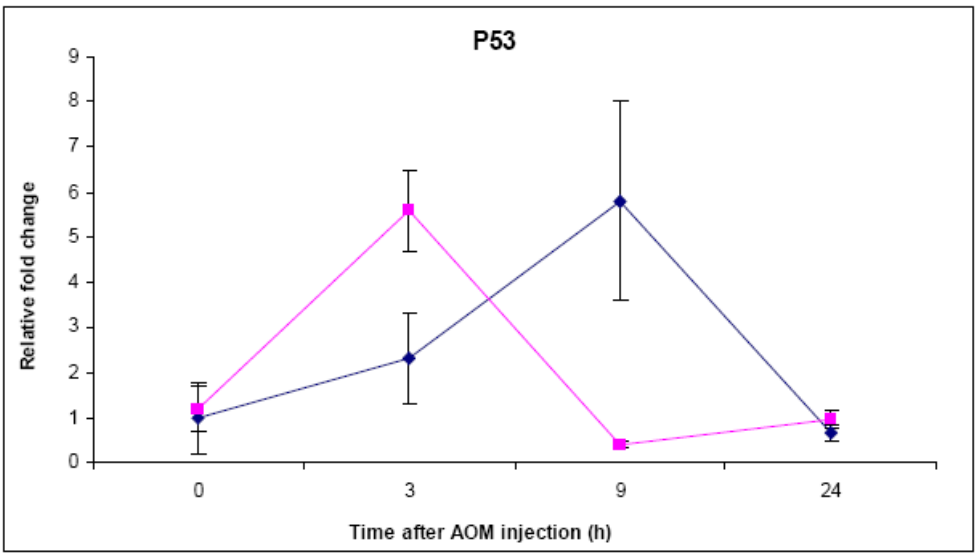

F
Bak
Ralative fold change
3.5
3
2.5
2
1.5
1
0.5
0
0
3
9
24
Time after AOM injection (h)

Figure 18. *P53* gene expression pattern in colon epithelium of Zucker rats after AOM injection

Obese and Lean Zucker rats were injected with AOM and terminated at three different time intervals. Time 0 indicates the control group that were not injected. The apoptotic regulatory P53 gene expression was assessed using quantitative Real-Time RT-PCR relative to the non-injected animals in each individual group (N=12) and normalized to the housekeeping gene *B-actin* as an internal control. Line graphs represent the mean ±S.E.M of the relative fold change of individual genes at four different time points in obese and lean colonic mucosa. Tissues were collected from 60 rats, 30 obese and 30 lean, and each individual gene expression was measured in triplicate. The asterisk (*) indicates a significant difference over the time ($P<0.05$). Blue graphs represent gene expression pattern in lean animals, while red graphs represent gene expression pattern in obese animals.


P53
Relative fold change
9
8
7
6
5
4
3
2
1
0
0
3
9
24
Time after AOM injection (h)

3.5 Hepatic BCL-2 family gene expression patterns in Zucker rats after AOM injection

In this part of the study, it was hypothesized that obesity would affect the BCL-2 family of gene expression patterns in obese individuals such that they show different apoptotic gene expression patterns than their lean counterparts within 24 hours of AOM injection. One way ANOVA statistical tests were performed to examine the overall differences in gene expression patterns between obese and lean animals followed by a Tukey HSD post hoc test to show the overall significant differences between four individual time points in both groups.

As described in Fig.19-A, obese and lean animals show roughly the same *Bcl-2* gene expression pattern in liver. However, the one way ANOVA shows significant difference between the two groups (P= 0.014). This supports the results of the Tukey HSD test, which revealed that there are significant differences in expression between three, nine and twenty four hours post AOM injection time points in both groups. In other words, at three hours after treatment, the *Bcl-2* gene expression is significantly higher than what it is at nine (P=0.034) and twenty-four hours (P=0.000). As seen in Fig.19-B, obese and lean Zucker rats show a similar profile of *Bcl-XL* expression pattern, but this pattern statistically is different in liver over twenty-four hours after AOM injection (P=0.00). The Tukey HSD test revealed that *Bcl-XL* gene expression at three hours post injection is significantly higher than what it is at zero and twenty four hours (P=0.012 and P=0.003 respectively) and at nine hours compare to what it is at zero and twenty four hours in both lean and obese animals (P=0.019 and P=0.005 respectively).

As seen in Fig.19-C, a one way ANOVA test showed that the overall *Bax* expression pattern in liver is significantly different between obese and lean Zucker rats within 24 hours after AOM injection, but its expression pattern profile seems very similar. In obese and lean individuals, *Bax* gene expression is significantly up-regulated after AOM administration, and a Tukey post hoc test revealed that in both obese and lean rats the *Bax* gene expression at time zero is significantly lower than what it is at three, nine, and twenty four hours after injection.

A one way ANOVA revealed that *Bad* gene expression pattern, within twenty-four hours after AOM injection, in obese animals is significantly different from their lean counterparts (P=0.02) with a peak at nine hours post injection in obese group. Also it seems that the *Bad* gene expression profile is diferent in obese and lean individuals. The Tukey HSD test does not show

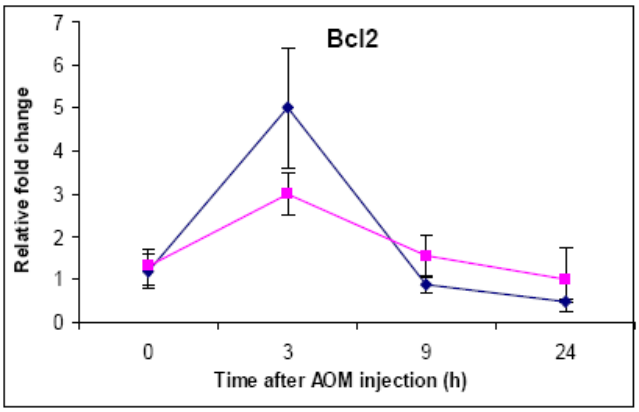
any significant difference between different individual time points (Fig.19-D). Moreover, *Bik* gene expression pattern, either by profile or statistically, does not show any difference in lean and obese animals as well as no significant change between different time intervals (Fig.19-E). *Bak* expression pattern, based on the overall ANOVA results, is significantly different in obese animals from their lean counterparts over 24 hours after AOM administration (P=0.02). In both obese and lean individuals *Bak* is up-regulated after AOM administration with the highest level at three hours post injection which is significantly higher than time zero (P=0.002)(Fig.19-F).

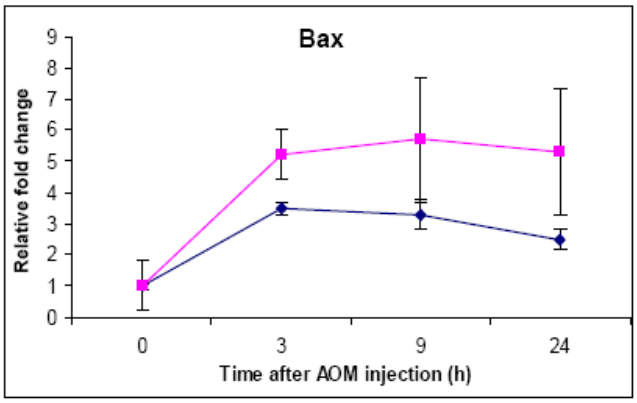
It seems that *Bad* is the only gene that's expression pattern profile is different in obese and lean individuals (Fig.19-D), and all the other genes, dynamically, follow the same expression profile in both groups. Interestingly, these profiles are different from one gene to another.

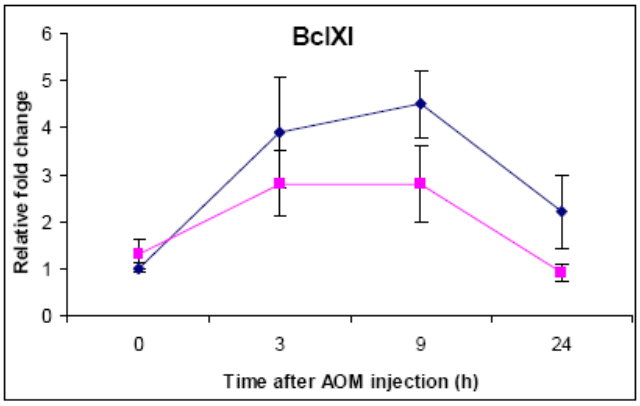
Fig.20 shows the *P53* gene expression pattern in liver. The ANOVA results indicated that, there is a significant difference between the overall *P53* gene expression pattern in obese and lean individuals within 24 hours post AOM injection (P=0.002). The dynamic pattern seems different between the individual time points as well. Also, in lean animals, *P53* is up-regulated after injection and reaches its expression peak at nine hours post injection and is then down-regulated by twenty four hours post injection. Further analysis applying the Tukey post hoc tests revealed that at nine hours post injection *P53* gene expression in both groups is significantly different from what it is at twenty four hours after injection (P=0.002).

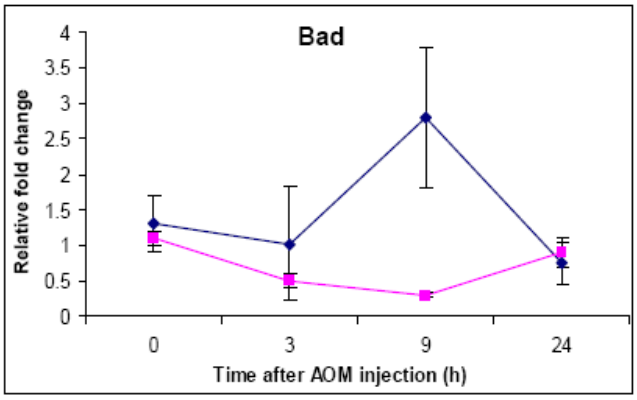
Figure 19. Anti- and pro-apoptotic gene expression pattern in liver tissue of Zucker rats after AOM injection

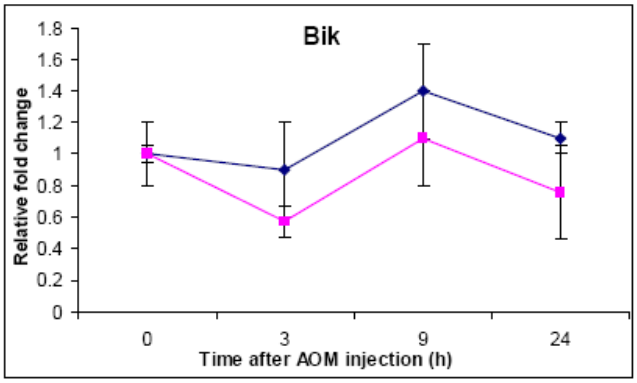
Obese and Lean Zucker rats were injected with carcinogen AOM and terminated at three different time intervals. Time 0 indicates the control group that were not injected. The anti-apoptotic and pro-apoptotic gene expressions were assessed using quantitative Real-Time RT-PCR relative to the non-injected animals in each individual group and normalized to the housekeeping gene *18srRNA* as an internal control. The anti-apoptotic *Bcl-2* and *Bcl-XL* results were presented in panel A and B, respectively, and pro-apoptotic *Bax, Bad, Bik*, and *Bak* gene expression patterns were presented in sections C, D, E, and F, respectively. Line graphs represent the mean ±S.E.M of the relative fold change of individual genes at four different time points in obese and lean colonic mucosa. Tissues were collected from 60 rats, 30 obese and 30 lean, and each individual gene expression was measured in triplicate. The asterisk (*) indicates a significant difference over the time ($P<0.05$). Blue graphs represent gene expression pattern in lean animals, while red graphs represent gene expression pattern in obese animals.


Bcl2
Relative fold change
Time after AOM injection (h)
0
3
9
24


Bax
Relative fold change
Time after AOM injection (h)
0
3
9
24


BclXl
Relative fold change
Time after AOM injection (h)
0
3
9
24


Bad
Relative fold change
Time after AOM injection (h)
0
3
9
24


Bik
Relative fold change
Time after AOM injection (h)
0
3
9
24

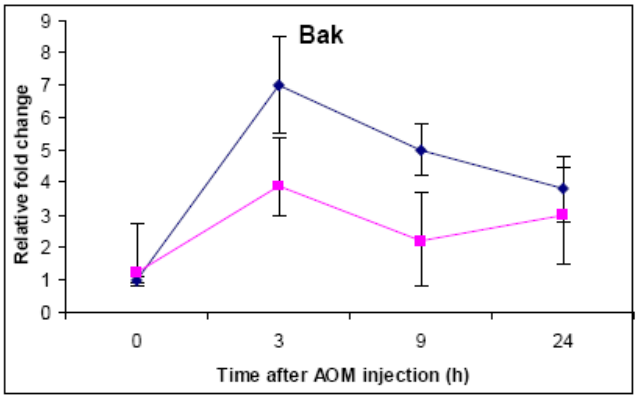
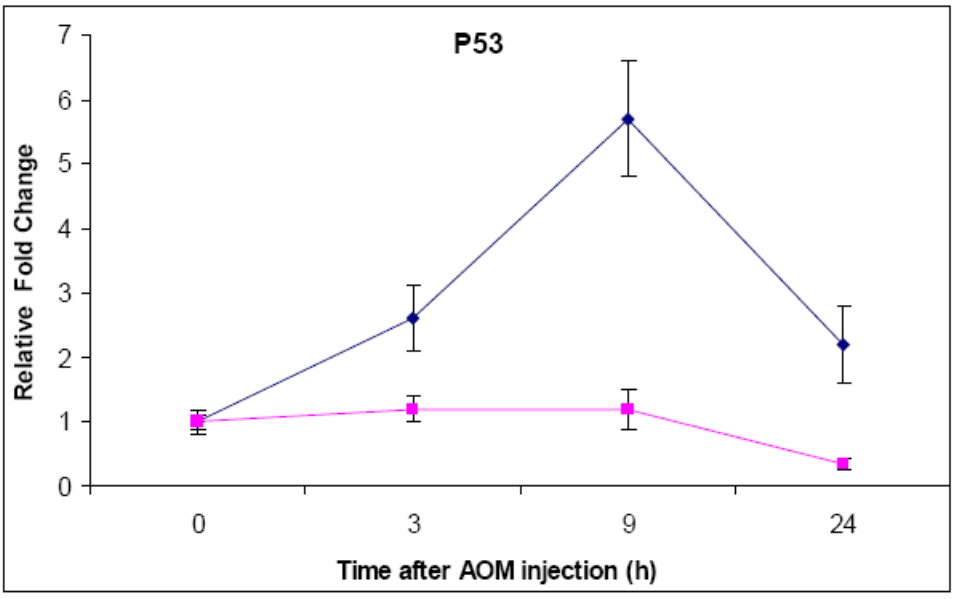

Bak
Relative fold change
Time after AOM injection (h)
0
3
9
24

Figure 20. *P53* gene expression pattern in liver tissue of Zucker rats after AOM injection

Obese and Lean Zucker rats were injected with AOM and terminated at three different time intervals. Time 0 indicates the control group that were not injected. The apoptotic regulatory P53 gene expression was assessed using quantitative Real-Time RT-PCR relative to the non-injected animals in each individual group (N=12) and normalized to the housekeeping gene *18srRNA* as an internal control. Line graphs represent the mean ±S.E.M of the relative fold change of individual genes at four different time points in obese and lean colonic mucosa. Tissues were collected from 60 rats, 30 obese and 30 lean, and each individual gene expression was measured in triplicate. The asterisk (*) indicates a significant difference over the time ($P<0.05$). Blue graphs represent gene expression pattern in lean animals, while red graphs represent gene expression pattern in obese animals.


P53
Relative Fold Change
7
6
5
4
3
2
1
0
0
3
9
24
Time after AOM injection (h)

Chapter 4 Discussion

In the first part of this study, some of pro-and anti apoptotic members of the BCL-2 family of genes were examined to determine if their expression level is altered in obese individuals, and if any of the differences found can explain why obese animals are more sensitive to the progression of colon cancer and liver diseases.

The second part of this study focused on the alteration of apoptotic related gene expression in the colonic epithelium and liver tissue of obese and lean rats within 24 hours of AOM- induced DNA damage.

Although, different studies have investigated the role of apoptosis and its alteration in colon cancer and hepatic steotasis in recent years, knowledge in this field is still limited. In particular, there is a gap in the current understanding of apoptotic gene expression levels in obese individuals. Expression of these genes may play a role in the increased incidence of variety of diseases including colon cancer and hepatic steotasis.

Apoptosis is a highly regulated process which is basically defined by morphological changes including chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. Apoptosis could be affected by tumor suppressor gene *P53*. In normal cells and in normal environment, P53 protein is rapidly degraded and stayed at very low levels within the cell. However, some of the stress signals, such as DNA damage, hypoxia, oncogene activation, and metabolic changes induce the rapid elevation of P53 mRNA and/or protein levels (*84*). As mentioned before, one of the most important functions of *P53* as a tumor suppressor gene is its ability to inhibit cell growth by inducing either cell cycle arrest or apoptosis. Furthermore, the function of P53 protein as a transcription factor contributes to both responses . Many cellular genes have been shown to be transcriptionally regulated by *P53*. These genes can be broadly divided into those involved in cell cycle arrest and those that contribute in the apoptotic response. It should be noticed that these two groups are separable (*4*). In fact, the choice of response to *P53* is strongly influenced by the cell type, the cellular environment, and the type of

signal. As an example, it was proposed that repairable low level DNA damage, which induces low levels of *P53*, leads to cell cycle arrest, while extensive and un-repairable DNA damage induces high levels of *P53* and leads to apoptosis (*85*). *P53*-induced apoptosis has been shown to regulate both *Bcl-2* and *Bax* expressions *in vivo* (*86*). Expression of the BCL-2 family of genes regulates cell survival by controlling apoptosis. *Bcl-2* and *Bcl-XL* genes and their encoded proteins are anti-apoptotic members, whereas others, such as *Bax, Bad, Bak* and *Bik* genes and their protein products are pro-apoptotic. The BCL-2 family of proteins are able to form homo- and/or hetero-dimers among themselves; their association and the relative ratio of pro- to anti-apoptotic genes and proteins are thought to be responsible for directing the cells toward death or survival. Current data have not clarified which dimers are critical regulators of apoptosis.

In addition, it is worthy of mention that oxidative stresses have a significant role in the development of many human diseases, such as cancer and liver dysfunctions (*87*), and different studies have shown the effective role of either natural or synthetic antioxidants as pharmaceutical agents to prevent and cure of these diseases (*88*). Oxidative stresses, in aerobic life, arise from both endogenous and exogenous sources such as higher level of glucose metabolism that in turn generate hydroxyl radicals and other reactive oxygen species (ROS), which as well as hyperglycemia trigger programmed cell death (*89*).

Zucker obese rats show hyperglycemia and subsequently more reactive oxygen species; therefore, it was expected that in these animals *P53* and its transcriptionally regulated apoptotic involving genes would show higher expression levels. It was hypothesized that obesity as a metabolic disorder and its consequent problems such as hyperinsulenemia, hyperglycemia, and higher level of ROS should up-regulate *P53* and make tissue more sensitive to apoptosis and disease progression either before or after AOM injection and DNA damage induction. Moreover, since obesity does affect the *P53* expression level, it could change the pro- and anti-apoptotic gene expression pattern of BCL-2 family.

4.1 Effects of obesity on apoptosis in colon tissue

Colon is a highly proliferative tissue with a constant turnover of cells. This organ is sensitive to tumor development, and it was documented that colorectal cancer is the third

most common cancer in both men and women including 9% of all cancer deaths in 2008 (Cancer Facts and Figures, Annual report of American Society of Cancer). Epidemiological data suggests that obesity is a risk factor for colon cancer . The animal model of human obesity, Zucker obese rats which were used in this study, has been proven to be more sensitive to carcinogen-induced colon cancer than their lean counterparts (*90, 91*). This study was carried out to examine if the obese genotype of the Zucker rat animal model alters the apoptotic gene expression prior to any disease development with and without carcinogen injection.

4.1.1 The effect of obesity on pro- and anti-apoptotic gene expression levels in colon tissue prior to AOM injection

A differential apoptotic gene expression study was performed on obese and lean Zucker rats by means of quantitative Real-Time RT-PCR to figure out the probable effect of obesity on apoptotic gene expression. The data in Fig.9 shows that expression of the pro-apoptotic *Bax, Bad* and *Bak* genes is significantly up-regulated in obese animals, while, anti-apoptotic *Bcl-2* gene expression is significantly down-regulated in obese individuals. The ratio of *Bcl-2/Bax* genes and/or proteins is one the most important indicators of whether a cell will survive or undergo apoptosis (*6, 97*), and it was shown that if this ratio is lower than one, then that particular cell undergoes apoptosis. In current study, this ratio in obese colon mucosa is about 0.1 which is lower than what it is in lean mocusa (1.25) and suggests that in the colon of obese individuals cells are more sensitive to apoptosis. This result confirms the previous results seen in adipose tissue of obese Zucker rats (*92*). The *P53* gene, which can work as either a cell survival factor or apoptotic activator did not show a significant change in expression level in both groups in colon mucosa. It has been previously reported that *Bcl-XL* gene expression is up-regulated by insulin-like growth factor-1/IGF-1 (*6*), which is higher in obese individuals (*93*). However, in this part of current study, *Bcl-XL* gene expression level was not changed significantly in obese animals. The anti-apoptotic *Bik* gene presents a widespread tissue distribution, and may be rapidly turned over during apoptosis (*47*). Also, its regulation takes place at the transcriptional level with over-expression of *P53*

(*94*). Current results can not confirm or decline the previous one since either *P53* or *Bik* gene expression levels has not changed in non-injected obese and lean animals (Fig.9).

Pro-apoptotic Bax protein induces apoptosis by permeating the mitochondrial membrane, allowing Cytochrome C release, initiating apoptosis through caspase activation and apoptosome complex formation. It was previously shown in our research group that elements of the sphingolipid pathway are altered in obese individuals without carcinogen injection and prior to cancer development. Also, applying Western blot and densitometric analysis, it was indicated that Bax protein is 100% up-regulated in obese colon tissue compared to lean. Furthermore, it was demonstrated that Bcl-2 protein is significantly down-regulated in obese Zucker rats (Burrows and Bird, unpublished data).

On the other hand, some of these candidate genes could show different reaction at protein level due to either post transcriptional or post translational changes that may arise. For instance, P53 is one of the most important proteins for determining cell fate. In fact, although it was expected that *P53* gene would show a higher expression level in obese individuals, current data indicate no change in its gene expression level. However, it is still possible that the protein expression level could be up-regulated in obese animals. Also, at the pro- and anti-apoptotic protein level, cell fate depends on the homo- and/or hetero-dimmers which would be oligomerized inside a cell.

As a conclusion, based on these results, the higher expression levels of *Bax* gene and protein and lower expression levels of *Bcl-2* gene and protein along with up-regulation of other pro-apoptotic genes in obese colon mucosa as well as the lower ratio of *Bcl-2/Bax* genes suggest that obese individuals are more sensitive to apoptosis and are more prone to colonic disease progression.

4.1.2 Early alteration of apoptotic gene expression levels in Zucker rat's colonic mucosa after AOM injection

Alteration of expression level of some apoptosis genes in colonic mucosa of Zucker obese and lean rats was estimated after exposure to azoxymethane (AOM) by means of quantitative Real-Time RT-PCR to find out the probable effect of obesity on the expression of these genes. The mechanism of cancer initiation by the carcinogen azoxymethane have been described (*95*). Activated metabolites of this agent form O6-

methylguanine in DNA, which induce the GC to AT transitions *in vitro* and *in vivo* (*96*) , that are associated with tumor initiation (*97*). Some of the damaged cells are repaired by internal repair systems, but some are not able to be repaired and will be eliminated by apoptosis; therefore, only a few numbers of surviving damaged cells have the opportunity to change to pre-cancerous cells. It was described that " in the early stage of experimental tumorogenesis, mitosis and nucleic acid synthesis is inhibited because of carcinogen-induced apoptosis and these alterations may induce cell cycle arrest and apoptosis after DNA damage" (*98*). However in late stages of DNA damage induction and carcinogen injection, mitosis is activated and depends on the ratio of cell division to apoptosis some cells change to pre-cancerous ones and then unbalanced cell proliferation and death causes tumorogenesis. Moreover, there are accumulating data suggest that apoptotic genes and proteins , such as those of the BCL-2 family, are associated with the pathogenesis of some types of malignancies (*99*). Therefore, amongst other cell death evaluating methods, studies on apoptotic gene expression seem valuable. In addition, it was previously reported that more epithelial cells are eliminated through apoptosis in distal part of the colon (left side of the body, 0-4 cm from the rectal end) than proximal (right side of the body, 8-16 cm from the rectal end) after carcinogen exposure. Also, AOM treatment causes profound damage to the distal colon in male Fisher rats, and only one AOM treatment provides more tumors in distal colon (*100*). This finding was confirmed in 2001 by Hong and his research group (*63*). Furthermore, in 1995 Bird *et. al.* explained that during experimental colon carcinogenesis, pre-cancerous cells appeared earlier and more rapidly in distal versus proximal colon and the amount of fat consumption did affect the tumorogenesis (*101*). It was also reported that majority of tumors in obese Zucker rats are located in distal region of colon tissue and obesity does directly affect the number of tumors, and in fact, "association between obesity and colon cancer is more often noticed in the distal rather than proximal colon" (Saxena and Bird, un-published data). Consequently, it seems that more molecular changes such as apoptosis and cell proliferation occur in distal colon after carcinogen exposure; therefore, in the current study, the distal colon has been studied. Based on the current results, three hours post AOM injection, *P53* and its downstream pro-apoptotic gene's expression levels significantly changed in obese animals. *P53, Bax*, and *Bad* genes show higher

expression, while *Bik* and *Bak* genes show lower expression level in obese individuals compare to their lean counterparts. As it was mentioned earlier, *P53* gene is responsible for different functions at the molecular level in cells, which can be classified by the type and amount of cellular stresses. Since a high level of DNA damage is usually induced by AOM injection, if the tumor suppressing function of *P53* is considered, higher expression levels of *P53* may result in less cell proliferation and more cell cycle arrest or more apoptosis. In addition, higher expression level of *P53* could explain up-regulation of its down-stream targets such as *Bax* and *Bad* in obese individuals. On the other hand, anti-apoptotic gene expression levels have not been changed at this time point in obese individuals compared to their lean counterparts, and the *Bcl-2/Bax* ratio in obese colon mucosa is 0.25, thus three hours after DNA damage induction obese individuals are more prone to apoptosis.

These results as expected agree with the observations of Hirose *et. al*. They found increasingly damaged cells with typical morphologic characters of apoptotic cells in distal part of colonic epithelium four hours after AOM treatment (*64*). Also, the same research group recorded that at eight hours after carcinogen treatment, the number of damaged cells as well as apoptotic bodies was increased and a significant decrease in the number of mitotic cells was observed. However, in the current study, it was noticed that at nine hours after DNA damage initiation, the apoptotic regulator gene *P53* and its downstream target pro-apoptotic *Bad* gene expression levels are significantly lower in obese individuals, while the pro-apoptotic *Bax* gene expression level is significantly higher at this time point in the obese group compared to their lean counterparts, and *Bak, Bik* and two anti-apoptotic genes follow the same expression level in both groups.

Although this part of results regarding *P53* expression level is not as expected (see Fig.12) the ratio of *Bcl-2/Bax* is still lower in obese colon and is equal to 0.05, which suggests that at nine hours post injection the colonic epithelial cells of obese individuals are more sensitive to cell death.

Hirose *et. al*. also explained that three days after AOM injection, the number of apoptotic cells were decreased (*64*); however, current study cannot confirm or decline the above data since one day after injection was the last time interval investigated. Refer to Fig.13 at 24 hours post AOM administration, pro-apoptotic genes, *Bax, Bad* and *Bik*,

show significantly higher expression levels in obese individuals and other genes follow the same expression level in both groups. Also, the *Bcl-2/Bax* ratio is 0.1 in obese animals which is lower than what it is in their lean counterparts. Therefore, it can be concluded that one day after DNA damage induction, obese individuals could be more sensitive to apoptosis and disease progression because of unbalanced cell death/proliferation. Previously, Hong and his collaborators using a "rat diet/colon tumorogenesis model system" figured out that they can follow tumor development in this system by looking at the early stages (0-12 hours) of tumorogenesis after carcinogen administration (*63*). Applying the TUNEL assay and a morphological study, this research group found that from time zero to nine hours post AOM injection, the apoptotic index initially increases in the colon epithelial cells and then decreases again up to 12 hours post injection. Also, they reported that in rats that were fed with high fat diet the apoptotic index increased up to nine hours and then declines, but in rats were fed with low fat diet the reaction of colon cells to AOM injection is different and apoptotic index simply increases over the time. Therefore, the apoptotic reaction of low fat diet animals within the first 12 hours after carcinogen administration was different from the high fat diet animals. *Bax* is the most important pro-apoptotic member of BCL-2 family that research on its expression level has been the focus of most of the apoptosis related studies. This gene and its protein product have the ability to inactivate the anti-apoptotic members and/or increase the activity of other pro-apoptotic members. In current study, *Bax* gene expression level is higher in obese animal with or without apoptotic signal that explain that why obese individuals could be more prone to apoptosis.

Regarding these results, the effect of obesity on overall pro- and anti-apoptotic gene expression patterns during the first twenty-four hours after carcinogen injection were examined statistically. In this part of results, obese and lean injected animals were compared to the non-AOM injected ones in their own group. In this way, the effect of carcinogen administration regardless of metabolic condition of animals was followed at three different time points. Then these effects were compared between the obese and lean groups to find out whether or not apoptotic gene expression patterns in obese individuals are statistically different from their lean counterparts. It was very interesting to find that the anti-apoptotic *Bcl-2* and *Bcl-XL* genes follow a similar expression pattern in obese

groups and are different from what it is noticed in lean individuals. Moreover, pro-apoptotic gene expression patterns in obese and lean animals are statistically different from each other. These results could explain that why obese colon is more susceptible to disease progression compare to its lean counterpart. As seen in figure 17, anti-apoptotic genes in obese animals show the maximum expression at three hours post AOM injection which is significantly higher than what it is at twenty four hours and suggest that one day after DNA damage induction, obese group show more trends to apoptosis.

All together, as it was hypothesized, within 24 hours of initial stages of AOM – induced colon tumorogenesis, obese individuals are more sensitive to DNA damage. Also, regarding the apoptotic gene expressions the reaction of obese and lean Zucker rats to carcinogen injection is statistically different, which confirms the previous results in a "rat diet/colon tumorogenesis model system" (*63*). However, still more investigation in this project, such as TUNEL assay, apoptotic protein expression analysis, and morphological studies at three or even more time intervals, is required to find out the effect of obesity on apoptotic index in colonic tissue after carcinogen administration.

4.2 Obesity effects apoptosis in liver tissue

Hepatocellular apoptosis is a key outcome of many forms of liver disorders and is triggered by signals that activate either the extrinsic apoptotic pathway through the induction of death receptor expression, or the intrinsic apoptotic pathway through up- and/or down-regulation of the BCL-2 family of genes. The consequences of programmed cell death are more noticeable as more hepatocytes are eliminated leading to liver dysfunction. In recent years, more understanding about apoptotic signalling processes and the genes and proteins involved has been achieved, so more efficient therapeutic control mechanisms might be available in the future to help combat this problem.

In liver tissue, dysregulation of programmed hepatocyte death such as failure to undergo apoptosis can cause severe problems ranging from autoimmune diseases to cancer, or a high incidence of apoptosis which contributes to the progression of pathological states including chronic liver diseases (*66*). Indeed, cells are divided in two types depending on the involvement of mitochondria in apoptosis (*35*). Hepatocytes are classified as the type II cells which mean that mitochondria play an essential role in the

regulation of apoptosis and are actually considered strategic centers in the control of cell death. Given that mitochondria plays critical role in hepatocellular apoptosis, the *P53* gene and some of its downstream genes such as BCL-2 gene family members are good candidates to be investigated.

Previously, it was reported that obese mice show hepatic steatosis (*102*). About 10 years later, it was documented that obese patients are more susceptible to acute liver failure when they are exposed to apoptotic signals(*103*). Moreover, *in vitro* studies using liver cancer cell lines have shown that free fatty acids promote up-regulation of the Fas/CD95 receptor and consequently activate the extrinsic apoptotic pathway (*77*). Also, it was reported recently that Sprague-Dawley rats were fed in high fat diet show more TNF-α, caspase3, and Bax mRNA expressions and no change in anti-apoptotic *Bcl-2* and *Bcl-XL* expressions compared to the control group (*104*). In current study, after tissue harvesting it was noticed that in Zucker rats the obese livers were significantly larger than the lean ones and appeared pale and creamy with lighter colour compared to the darker red colour of their lean counterparts. Furthermore, it was previously shown in our research group that obese animals have hepatic steotasis characterized by lipid accumulation within the hepatocytes, which confirms the results in other animal models, while the lean liver shows normal histology (*105*).

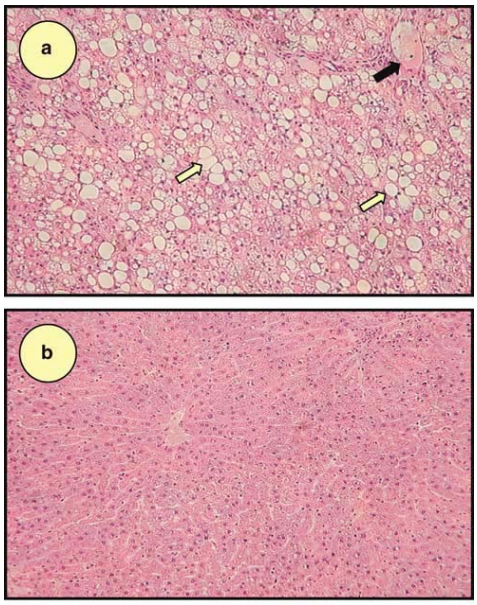


Figure 21. Histology of obese and lean liver in Zucker rats

Representative liver histology, magnification _100: a segment of liver was fixed in a buffered formalin solution and was stained with haematoxylin (staining the nuclei black) and eosin (staining the cytoplasm pink). (a) liver tissue from Zucker obese rat, yellow arrows show variable sizes of lipid droplets in hepatocytes; (b) liver tissue of Zucker lean rat shows normal liver architecture.

The expression level of the most important pro- and anti-apoptotic genes of the intrinsic apoptotic pathway were assessed in order to determine if the expression of these genes was altered in the liver of obese individuals before and after AOM administration. As some of these genes are regulated transcriptionally by the tumor suppressor *P53*, it was valuable to see if any alterations occurred at the gene expression level.

Results of current study show that before DNA damage induction through carcinogen injection, apoptotic regulator gene *P53* and its downstream targets, anti-apoptotic *Bcl-XL* as well as pro-apoptotic *Bax* and *Bak* are significantly up-regulated in the liver of obese individuals. Pro-apoptotic *Bad* and *Bik* show similar expression level in obese and lean animals. Perhaps, if the transcriptional regulatory activity of *P53* is considered in this case, a higher expression level of *P53* could be correlated with different incidence of apoptosis and/or hepatic tumor formation in obese animals. As it was mentioned earlier, *Bcl-XL* expression is up-regulated by higher level of insulin-like growth factor-1 (IGF-1) (*6, 93*) in obese animals. Anti-apoptotic *Bcl-2* gene expression level in obese animals is not different from their lean counterparts and confirms the previous results in Sprague-Dawley rats fed with high fat diet (*104*).

As mentioned earlier, obesity correlates with higher oxidative stress due to hyperglycaemia. Furthermore, liver steatosis is closely associated with liver cell death. Increased expression of death receptors in obese livers sensitizes these cells to pro-apoptotic signals, consequently triggering excessive hepatocyte apoptosis and inflammation (*77*). Also, in our research group applying Western blot and densitometric analysis in two different projects, it was shown that Bax protein is up-regulated by approximately 100%, and Bcl-2 protein expression level is approximately 75% lower in

obese Zucker rats compare to their lean counterparts (Naahidi and Bird, Burrows and Bird, unpublished data).

Due to prolonged oxidative stresses and steatosis, it was hypothesized that obese individuals are more sensitive to apoptosis. Current results approve this hypothesis and are in accord with previous studies; since, over expression of pro-apoptotic genes along with a higher expression level of *P53* and a lower *Bcl-2/Bax* ratio in obese individuals, which is 0.4 compare to 1.2 in their lean counterparts, suggest that obese liver tissue is more prone to programmed cell death and disease progression.

In the next part of this study Zucker obese and lean rats were exposed to AOM carcinogen to induce DNA damage and apoptosis to investigate the hypothesized alteration of some apoptotic gene expression levels in liver tissue applying quantitative Ral-Time RT-PCR. At three hours post injection, as expected, the tumor suppressor gene *P53* and its downstream transcriptional target genes *Bax* are significantly up-regulated in obese individuals. Anti-apoptotic and other pro-apoptoticgenes show the same expression levels in both groups of animals. A higher expression level of *P53* along with a lower *Bcl-2/Bax* ratio in obese individuals (0.3 in obese compare to 1.3 in lean animals) may indicate more sensitivity of obese animals to DNA damage in the first three hours of apoptosis induction. However, at 9 hours after injection, *Bax* is the only gene that its expression level is changed in obese individuals. *Bax* is up-regulated in obese animals while the other genes unchanged. The *Bcl-2/Bax* ratio is 0.12 in obese individuals, which suggests at nine hours post injection obese individual's liver cells are still more prone to apoptosis. Pro-apoptotic *Bax, Bad, Bak* and anti-apoptotic *Bcl-2* gene expression levels are significantly higher in obese animals at 24 hours after DNA damage. The only part of current results that confirm the previous data about the effect of higher IGF level of obese animals on *Bcl-XL* higher expression level is achieved in non-injected animals and results after AOM injection decline that. In fact, it seems that after carcinogen injection, the effect AOM-induced apoptotic signals are more important and more effective than higher IGF level in obese individuals because after apoptotic signal induction, the anti-apoptotic *Bcl-XL* gene expression level did not changed in obese animals compared to their lean counterparts.

The effect of obesity on overall pro- and anti-apoptotic gene expression patterns during the first twenty-four hours after carcinogen injection was statistically examined as well. Obese and lean injected animal were compared to the non-injected ones within their own group in order to follow the effect of AOM administration and DNA damage induction without considering the effect of obesity as a metabolic disorder. The results indicate that the *Bcl-2, Bcl-XL, Bax,* and *Bik* gene expression profiles are roughly similar in both groups; however the overall anti-apoptotic *Bcl-2* and *Bcl-XL* as well as pro-apoptotic *Bax* gene expression patterns are different in obese animals from their lean counter parts over the first twenty-four hours after AOM injection. Also, *P53, Bad,* and *Bik* gene expression patterns were significantly changed in obese individuals. As expected, after carcinogen injection, obese individuals show a different reaction to DNA damage from their lean counterparts. Consistently, the altered levels of pro- and anti-apoptotic gene expressions in liver tissue at three different time intervals post AOM injection, as well as lower *Bcl-2/Bax* ratio in obese individuals suggest an increase in pro-apoptotic signals in obese liver tissue, which in turn would explain the reason that why these animals are more susceptible to liver disease progression such as liver steatosis.

Current results along with previous results in our research group not only support our hypothesis, also confirm previous studies reporting that steatotic livers are primed for apoptosis (*103*). As there are many other genes and proteins involved in mediating apoptosis, further investigation, such as assessing the pro- and anti-apoptotic protein expression level and morphological studies, is required to investigate the exact apoptotic index and the role of these genes and proteins in triggering apoptosis in steatotic liver.

4.3 Apoptotic gene expression differences between liver and colon

Liver and colon tissues possess completely different histology and functions. Previously it was shown that AOM injection cause DNA damage in colon and liver, which is known to induce tumors in these tissues, and it was hypothesised that the formation and persistence of methylated DNA during replication may be responsible for the initiation of tumors (*106*). On the other hand, it was noticed that after AOM injection, in rat model, the initial DNA damage is higher in liver compared to colon, although liver

tumors have not been reported to be produced by a single dose of the carcinogen. (*63*). Also, after carcinogen administration, DNA damage persists in rat colon, but not in rat liver, which indicates the grater ability of liver to remove damaged DNA either through apoptosis or repair the damaged DNA (*106*). Consistent with these results, in the last part of this study, it was hypothesized that the reaction of obese and lean Zucker rats to AOM induced apoptotic gene expression is different in liver and colon tissues.

As seen in Fig.17-C and 19-C, applying two way ANOVA statistical method, the overall pro-apoptotic *Bax* gene expression pattern in liver is not significantly different from colon tissues of obese and lean Zucker rats over 24 hours post AOM injection (P= 0.36). Overall *Bik* gene expression pattern in colon and liver show significant differences (P=0.04) without a meaningful difference at each specific time point (Fig.17-E and Fig. 19-E). Also, *Bak* gene expression pattern is significantly different over 24 hours post injection between the two tissues (P=0.007) (Fig.17-F and 19-F). Furthermore, refer to figures 17 and 19 panels D, which show the *Bad* gene expression patterns in colon and liver, respectively, this pro-apoptotic gene did not show significantly different expression patterns in these particular tissues, since based on the two-way ANOVA test the P value is 0.18 with 95% confidence interval. A statistical comparison between results of Fig.17-B and 19-B indicated that anti-apoptotic *Bcl-XL* gene expression pattern in colon tissue is not significantly different from liver. The same result was achieved for the anti-apoptotic *Bcl-2* with no significant difference in its overall gene expression patterns in liver and colon (Fig.17-A and 19-A), while *P53* gene expression pattern is significantly different in liver and colon tissue within 24 hours after carcinogen injection (P=0.006) (Fig.18 and Fig.20).

In fact, it seems that the ability to remove or repair the initial damage is probably more important than the damage itself in determining the increase and/or decrease in either the tumor development or apoptosis index. Hong *et. al.* suggested that a higher apoptotic index is observed in liver within the first 12 hours post AOM injection in mice model (*63*). In current study, *P53* and its pro-apoptotic down-stream target genes *Bax, Bik, Bak*, and *Bad* expression levels are significantly different between colon and liver tissues within 24 hours after AOM injection in both lean and obese Zucker rats. Interestingly, anti-apoptotic *Bcl-2* and *Bcl-XL* genes did not show a significant difference in these two

tissues. As expected, in colon and liver tissues the apoptotic index could be different, since pro-apoptotic gene expressions are different, and actually it seems that the tumor suppressor *P53* and pro-apoptotic gene expression levels in liver tissue are higher than what it is colon within twenty four hours post AOM injection. Therefore, current results in Zucker rats confirm the previous results in other animal models and suggest that after apoptotic signal, liver tissue could be more prone to cell death. However, other factors should be considered to explain the grater sensitivity of colon to AOM induced tumorogenesis compared to liver. One important factor is the higher rate of cell turnover in colon, which would increase the chance that DNA synthesis would occur while the carcinogen-induced damage has not been repaired yet. Therefore, persistence and accumulation of damaged DNA through replication must be the initiator of tumorogenesis in colon tissue compared to liver. Therefore, more investigation on genes and proteins that are involved in cell proliferation and DNA repair system as well as applying other techniques such as morphological studies and TUNEL assay to show the real apoptosis index in these two tissues is required.

This was the first study comparing the expression level of BCL-2 family of genes in obese versus lean liver and colon tissue. As described earlier, during initial stages of carcinogen-induced DNA damage, apoptotic index increases in order to remove the cells with damaged DNA along with other repair mechanisms. However, in some tissues with grater cell turnover rate such as colonic mucosa, some cells continue DNA replication and proliferation while their genomic pool has not been repaired, although the rate of cell death has been increase because of carcinogen injection. Accumulation of damaged DNA, accompanied by mutations in late stages of carcinogen administration leads to tumorogenesis. On the other hand, in some organs such as liver, where the cell proliferation rate is not high, keeping the tight balance between cell proliferation and death is critical in their normal function. Therefore, more apoptosis compared to cell proliferation makes the tissue more susceptible to disease progression. In this study, it was shown that an obese genotype affects pro- and anti-apoptotic gene expression levels and patterns whether or not DNA damage has been induced in these tissues. The results show a clear alteration in apoptotic gene expression levels in obese animals compared to

their lean counterparts leading to the proposal that apoptosis may be involved in the obesity related colon and liver pathogenesis. Moreover, differential apoptotic gene expression analysis is one of the techniques of measuring cell death rate. However, in some cases, due to post translational and transcriptional modifications, apoptotic proteins may present a different expression level from genes. Also, pro- and anti-apoptotic protein conformations may affect the apoptosis rate as well. Therefore, it is better to apply other complementary techniques to find the accurate apoptotic index in apoptosis related studies.

APPENDIX A

Rightslink Printable License

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Dec 02, 2008

This is a License Agreement between Nafiseh Nafissi ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number	2027930023380
License date	Sep 14, 2008
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature
Licensed content title	The biochemistry of apoptosis
Licensed content author	Michael O. Hengartner
Volume number	407
Issue number	6805
Pages	pp770-776
Year of publication	2000
Portion used	Figures / tables
Requestor type	Student
Type of Use	Thesis / Dissertation
PO Number	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the

print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

APPENDIX B

Rightslink Printable License

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Dec 02, 2008

This is a License Agreement between Nafiseh Nafissi ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number	2080820026344
License date	Dec 02, 2008
Licensed content publisher	Nature Publishing Group
Licensed content publication	International Journal of Obesity
Licensed content title	Alleviation of hepatic steatosis accompanied by modulation of plasma and liver TNF-[alpha] levels by Trigonella foenum graecum (fenugreek) seeds in Zucker obese (fa//fa) rats
Licensed content author	J Raju and R P Bird
Volume number	30
Issue number	8
Pages	
Year of publication	2006
Portion used	Figures / tables
Requestor type	Student
Type of Use	Thesis / Dissertation
PO Number	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any

electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

REFERENCES

1. Hengartner, M. O. (2000) *407*, 770-776.
2. Currie, K., J. F., Wyllie, A. H. (1972) *British Journal of Cancer 26*, 239–257.
3. Currie, A. R., Wyllie, A. H., Kerr, J. F. (1980) *International Review of Cytology 68*, 251-306.
4. Yin, X.-M., and Dong, Z. (2003) *Essentials of Apoptosis*
A Guide for Basic and Clinical Research, Vol. 2, Humana Press Inc., Totowa, New Jersey.
5. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) *Cell 88*, 347–354.
6. Thomadaki, H. a. S., A. (2006) *Critical Reviews in Clinical Laboratory Sciences 43*, 1-67.
7. Horvitz, H. R. (1999) *Cancer Res 59*, 1701s-a-1706.
8. Wong, K., and Hengartner, M. (2005) in *Neuroacanthocytosis Syndromes* pp 187-195.
9. Cohen, G. M. (1997) *Biochemistry Journal 326*, 1-16.
10. Nicholson, D. W. a. T., N. A. (1997) *Trends in Biochemical Science 8*, 299–306.
11. Earnshaw, C. W., Martins, M. L., and Kaufmann, H. S. (1999) *Annual Review of Biochemistry 68*, 383–424.
12. Elmore, S. (2007) *Toxicol Pathol 35*, 495-516.
13. Rao, L., Perez, D. & White, E. (1996) *Journal of Cell Biology 135*, 1441-1445.
14. Wyllie, A. H. (1980) *Nature 284*, 555–556.
15. Nagata, S. (2000) *Experimental Cell Research 256*, 12-18.
16. Sakahira, H., Enari, M. & Nagata, S. (1998) *Nature 391*, 96–99.
17. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) *J. Biol. Chem. 272*, 17907-17911.
18. Meier, P., Finch, A., and Evan, G. (2000) *407*, 796-801.
19. Debatin, K.-M. (2004) *Cancer Immunology, Immunotherapy 53*, 153-159.
20. Esposti, M. D., Erler, J. T., Hickman, J. A., and Dive, C. (2001) *Mol. Cell. Biol. 21*, 7268-7276.
21. Hardwick, J. M. (2001) *Cell Death and Differentiation 8*, 109-110.
22. Nguyen, M., Millar, D., Yong, V., Korsmeyer, S., and Shore, G. (1993) *J. Biol. Chem. 268*, 25265-25268.
23. Puthalakath, H. a. S., A. (2002) *Nature 9*, 505-512.
24. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) *Molecular Cell 8*, 705–711.
25. Korsmeyer, D. T. C. a. S. J. (1998) *Annual Review of Immunology 16*, 395-419.
26. Ke, N., Godzik, A., and Reed, J. C. (2001) *J. Biol. Chem. 276*, 12481-12484.
27. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000) *Genes Dev. 14*, 2060-2071.
28. Kirkin V, J. S., Zornig M. (2004) *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1644*, 229-249.

29. Honoki, K., Tsujiuchi, T., Tsutsumi, M., Kido, A., Morishita, T., Yoshimoto, M., Miyauchi, Y., Mii, Y., Tamai, S., and Konishi, Y. (2000) *Toxicol Pathol 28*, 575-579.
30. Cory, S., Adams JM. (2002) *Nature Review in Cancer 2*, 647–656.
31. Gonzalez, G., Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G. (1994) *Development 120*, 3033–3042.
32. Minn, A., Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M, Thompson CB. (1997) *Nature 385*, 353–357.
33. Hu, Y., Benedict, M. A., Wu, D., Inohara, N., and Nunez, G. (1998) *Proceedings of the National Academy of Sciences 95*, 4386-4391.
34. Van Houten, N., Blake, S., Li, E., Hallam, T., Chilton, D., Gourley, W., Boise, L., Thompson, C., and Thompson, E. (1997) *Int. Immunol. 9*, 945-953.
35. Srivastava, R. (2007) *1*.
36. Apte, S., Mattei, MG., Olsen, BR. (1995) *Genomics 26*, 592-594.
37. Miyashita, T., Krajewski, S., Krajewska, M., Wang, HG., Lin, HK., Liebermann, DA., Hoffman, B., Reed, JC. (1994) *Oncogene 9*, 1799-1805.
38. Suzuki, M., Youle RJ, Tjandra N. (2000) *Cell 103*, 645-654.
39. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev. 13*, 1899-1911.
40. Ulrich, E., Kauffmann-Zeh A, Hueber AO, Williamson J, Chittenden T, Ma A, Evan G. (1997) *Genomics 44*, 195-200.
41. Herberg, J., Phillips S, Beck S, Jones T, Sheer D, Wu JJ, Prochazka V, Barr PJ, Kiefer MC, Trowsdale J. (1998) *Gene 211*, 87-94.
42. Leu, J., Dumont P, Hafey M, Murphy ME, George DL. (2004) *Nature Cell Biology 6*, 443-450.
43. Sattler, M., Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. (1997) *Science 275*, 983-986.
44. Petros, A., Olejniczak ET, Fesik SW. (2004) *Biochimica et biophysica acta 1644*, 83-94.
45. Kaipia, A., Hsu, S. Y., and Hsueh, A. J. W. (1997) *Endocrinology 138*, 5497-5504.
46. Verma, S., Budarf, ML., Emanuel, BS., Chinnadurai, G. (2000) *Gene 254*, 157-162.
47. Marshansky, V., Wang, X., Bertrand, R., Luo, H., Duguid, W., Chinnadurai, G., Kanaan, N., Vu, M. D., and Wu, J. (2001) *J Immunol 166*, 3130-3142.
48. Bird, R. P. (1998) in *Tumor Marker Protocols* (Hanausek, M., and Walaszek, Z., Eds.) pp 500, Humman Press.
49. Sinicrope, F., Roddey, G., McDonnell, T., Shen, Y., Cleary, K., and Stephens, L. (1996) *Clin Cancer Res 2*, 1999-2006.
50. Shanmugathasan, M., and Jothy, S. (2000) *Pathology International 50*, 273-279.
51. Lengauer C, K. K., Vogelstein B. (1998) *Nature 396*, 643-649.
52. Boyle, P., and Langman, J. S. (2000) *BMJ 321*, 805-808.
53. Harada K, I. S., Miyashita T, Osuga J, Yagyu H, Ohashi K, Yazaki Y, Yamada N. (1997) *FEBS letters 411*, 63-66.

54. Laudanski J, C. L., Niklinska WE, Kretowska M, Furman M, Sawicki B, Niklinski J. (1999) *Neoplasma 46*, 25-30.
55. Schorr, K., Li, M., Krajewski, S., Reed, J. C., and Furth, P. A. (1999) *Journal of Mammary Gland Biology and Neoplasia 4*, 153-164.
56. HUANG, S.-T. J., and CIDLOWSKI, J. A. (2002) *FASEB J. 16*, 825-832.
57. MacCarthy-Morrogh, L. W., L.; Brimmell, M.; Johnson, PW.; Packham, G. (2000) *Oncogene 19*, 5534-5538.
58. Biroccio, A., Benassi, B., D'Agnano, I., D'Angelo, C., Buglioni, S., Mottolese, M., Ricciotti, A., Citro, G., Cosimelli, M., Ramsay, R. G., Calabretta, B., and Zupi, G. (2001) *Am J Pathol 158*, 1289-1299.
59. Hattori, T., Ookawa, N., Fujita, R., and Fukuchi, K. (2000) *Acta Oncologica 39*, 495-500.
60. Li, X., Marani, M., Yu, J., Nan, B., Roth, J. A., Kagawa, S., Fang, B., Denner, L., and Marcelli, M. (2001) *Cancer Res 61*, 186-191.
61. Kondo, S., Shinomura, Y., Miyazaki, Y., Kiyohara, T., Tsutsui, S., Kitamura, S., Nagasawa, Y., Nakahara, M., Kanayama, S., and Matsuzawa, Y. (2000) *Cancer Res 60*, 4328 - 4330.
62. Zou, Y., Peng, H., Zhou, B., Wen, Y., Wang, S.-C., Tsai, E.-M., and Hung, M.-C. (2002) *Cancer Res 62*, 8-12.
63. Hong, M. Y., Chapkin, R. S., Morris, J. S., Wang, N., Carroll, R. J., Turner, N. D., Chang, W. C. L., Davidson, L. A., and Lupton, J. R. (2001) *Carcinogenesis 22*, 1831-1835.
64. Hirose, Yoshimi, Makita, H., Hara, A., Tanaka, N., and Mori. (1996) *Japanese journal of cancer research 87*, 575-582.
65. Hirose, Y., Yoshimi, N., Suzui, M., Kawabata, K., Tanaka, T., and Mori, H. (1997) *Mol Carcinog 19*, 25-30.
66. Dufour, J.-F., and Clavien, P.-A. (2005), Springer, Berlin.
67. Negro, F. (2005) *N Engl J Med 353*, 2200-2201.
68. Mehta, K., Van Thiel, D. H., Shah, N., and Mobarhan, S. (2002) *Nutrition Reviews 60*, 289-293.
69. Jiang, J., and Torok, N. (2008) *Metabolic syndrome and related disorders 6*, 1-7.
70. Fitzgerald, M. (2007) *The Nurse Practitioner 32*, 24-25.
71. Browning, J. D., and Horton, J. D. (2004) *The Journal of Clinical Investigation 114*, 147-152.
72. Takehara, Tatsumi, T., T, S., EB, R., L, H., M, J., T, M., Y, K., and N, H. (2004) *Gastroenterology 127*, 1189-1197.
73. Canbay, A., Feldstein, A., Baskin-Bey, E., Bronk, S. F., and Gores, G. J. (2004) *J Pharmacol Exp Ther 308*, 1191-1196.
74. Nanji, A. (2004) *Clinics in Liver Disease 8*, 559-574.
75. Javier Crespo, A. C., Pedro Fernández-Gil, Manuel Hernández-Guerra, Marta Mayorga, Agustín Domínguez-Díez, José Carlos Fernández-Escalante, Fernando Pons-Romero,. (2001) *Hepatology 34*, 1158-1163.
76. FELDSTEIN, A., CANBAY, A., GUICCIARDI, M., HIGUCHI, H., BRONK, S., and GORES, G. (2003) *Journal of hepatology 39*, 978-983.
77. Canbay, Kip, Kahraman, Gieseler, Nayci, and Gerken. (2005) *The Turkish Journal of Gastroenterology 16*, 1-6.

78. Guicciardi, M. E., and Gores, G. J. (2006) in *Fas Signaling* pp 103-117.
79. Jiang, W. (2006) *Critical Reviews in Oncology/Hematology 27*, 179-209.
80. Feldstein, A. E., and Gores, G. J. (2005) *Frontiers in Bioscience 10*, 3093-3099.
81. Zucker, L. M., and T. F. Zucker. (1961) *Journal of Heredity 52*, 275-278.
82. Zucker, T. F., and L. M. Zucker. (1962) *Experimental Biology and Medicine 110*, 165-171.
83. Zucker, L. M., and H. N. Antoniades. (1972) *Endocrinology 90*, 1320-1330.
84. Ashcroft, M., Kubbutat, M. H. G., and Vousden, K. H. (1999) *Mol. Cell. Biol. 19*, 1751-1758.
85. Vousden, K. H. (2006) *J Cell Sci 119*, 5015-5020.
86. Reed, J. (1994) *J. Cell Biol. 124*, 1-6.
87. Parola, M., and Novo, E. (2005) *Journal of Hepatology 43*, 1096-1097.
88. Frei, B. (2004) *J. Nutr. 134*, 3196S-3198.
89. Valkoa, M., Leibfritzb, D., Moncola, J., Croninc, M. T. D., Mazura, M., and Telser, J. (2007) *The international journal of biochemistry & cell biology 39*, 44-84.
90. Weber, R. V., Stein, D. E., Scholes, J., Kral, J.G. (2000) *Digestive diseases and sciences 45*, 890-895.
91. Raju, J., and Bird, R. P. (2003) *Cancer Res 63*, 6595-6601.
92. Briscini, L., Tonello, C., Dioni, L., Carruba, M. O., and Nisoli, E. (1998) *FEBS Letters 431*, 80-84.
93. Giovannucci, E. (1995) *Cancer Causes and Control 6*, 164-179.
94. Mathai, J. P., Germain, M., and Shore, G. C. (2005) *J. Biol. Chem. 280*, 23829-23836.
95. Saffhill, R., Margison, G. P., and O'Connor, P. J. (1985) *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 823*, 111-145.
96. Loechler, E. L., Green, C. L., and Essigmann, J. M. (1984) *Proceedings of the National Academy of Sciences of the United States of America 81*, 6271-6275.
97. Herron, D. C., and Shank, R. C. (1982) *Carcinogenesis 3*, 857-860.
98. Klaunig, J. E., Kamendulis, L. M., and Xu, Y. (2000) *Human and Experimental Toxicology 19*, 543-555.
99. Korsmeyer, S. (1992) *Blood 80*, 879-886.
100. Yoshinobu Hirose, N. Y., Hiroki Makita, Akira Kara, Takuji Tanaka, Hideki Mori,. (1996) *Cancer Science 87*, 575-582.
101. Lasko, C., and Bird, R. (1995) *Cancer Epidemiol Biomarkers Prev 4*, 49-55.
102. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, and JM., F. (1995) *Science 269*, 543-546.
103. Canbay, A., Chen, S., Gieseler, R., Malago, M., Karliova, M., Gerken, G., Broelsch, C., and Treichel, U. (2005) *Hepatogastroenterology 52*, 1516-1520.
104. Wang, Y., Ausman, L. M., Russell, R. M., Greenberg, A. S., and Wang, X.-D. (2008) *J. Nutr. 138*, 1866-1871.
105. Raju, J., and Bird, R. P. (2006) *30*, 1298-1307.
106. Rogers, K. J., and Pegg, A. E. (1977) *Cancer Res 37*, 4082-4087.