

## **Chapter 1**

# **Introduction**

Improvements in technology lead to a sedentary lifestyle and consequently, an increase in body weight (Goldstein and Scalia, 2004). Obesity is associated with a high fat (HF) diet and this circumstance subsequently induces insulin resistance which is a major risk factor for diabetes and cardiovascular diseases (Kadowaki and Yamauchi, 2005). Type 2 diabetes mellitus (T2D), which in earlier times was called non insulin dependent diabetes, is nowadays one of the most common disarrays. It is known that T2D is a metabolic disorder that is noticeable through increased levels of plasma glucose ahead of the normal physiological range and is characterized by a progressive loss of glycemic control (Winter *et al.*, 2006).

One of the most important proteins which plays a basic role in insulin sensitivity, blood glucose and lipids, is adiponectin. Studies leading to the knowledge of the exact mechanism and effects of this protein on blood glucose and lipid profile can lead to the reduction in risk of type 2 diabetes.

Adiponectin is a protein which was originally identified in 1995 by Scherer (Scherer *et al.*, 1995). It is encoded by the *AdipoQ* gene in humans, and is one such adipokine that has recently attracted much attention (Kadowaki *et al.*, 2006; Capeau, 2007). Although adiponectin appears to exist as a full length protein in plasma, it can also exist as smaller, globular fragments as was reported by Lodish's group (Shapiro & Scherer, 1998; Kadowaki & Yamauchi, 2005). It is assumed that adiponectin is totally secreted by the adipose tissue. Although its mechanisms of action, active forms, receptors and signalling pathways remain, so far, incompletely understood ; some beneficial roles of this protein such as improved insulin sensitivity, glucose tolerance and lipid profile, have been identified (Capeau, 2007). It is interesting to note that Matthias B. Schulze *et al.*, suggested that increased adiponectin levels might be associated with better inflammation reduction in diabetic subjects (Schulze *et al.*, 2004). The risk factors including obesity and diabetes affect our arteries. Since cholesterol deposits in the arteries of patients with type 2 diabetes are much higher, an increase in adiponectin levels might be a helpful target for decreasing the atherosclerotic risk present in diabetic (Schulze *et al.*, 2004).

According to the anti-inflammatory properties of adiponectin, the effects of this protein on other inflammations such as gastric ulcer which is one of the common gastrointestinal diseases that leads to treatment costs of up to millions of dollars annually, is worth studying. Gastric ulcer occurs when the lining of organs is corroded by the acidic digestive juices which are secreted by the stomach cells.

A number of agents are available for the treatment of diabetes (Natali and Ferrannini, 2006) and gastric ulcer. Among them, Metformin and Omeprazole are two common drugs for the treatment of the diseases listed above, respectively.

It is a proton pump inhibitor which is used to treat symptoms of gastric ulcer. Omeprazole is a substituted benzimidazole which inhibits acid secretion by acting on

the hydrogen-potassium exchanger ( $H^+K^+$ -ATPase) for the apical plasma membrane of the gastric mucosa (Sato *et al.*, 1989).

On the other hand, Metformin is an oral diabetes medicine that is considered an insulin sensitizer controlling blood sugar levels by decreasing the liver uptake of lactate. It belongs to the biguanide class (dimethylbiguanide) of anti-diabetic drugs and is the most widely prescribed medicine for the treatment of type 2 diabetes mellitus (Basu *et al.*, 2008). Metformin suppresses endogenous glucose production and also helps diabetic patients lose weight or, at slightest, keep their weight stable (Levri *et al.*, 2005; Winter *et al.*, 2006). It lowers glucose concentrations in diabetic patients by increasing glucose uptake and decreasing glucose production. Although the precise mechanisms of biguanides are not entirely definite, they are mostly attributed to an increase in nonessential tissue sensitivity to insulin, reduction in hepatic gluconeogenesis, and decrease in intestinal glucose absorption (Bailey, 2008; Basu *et al.*, 2008).

Since adiponectin plays an important role in the suppression of the metabolic disarrangement that may result in type 2 diabetes and the levels of adiponectin being reduced in diabetics compared to non diabetics a combination of this protein with a common medicine such as metformin would potentially be beneficial as a treatment.

The specific objectives of the present study include:

- Examination and characterization of recombinant adiponectin in *P. pastoris*.
- Evaluation of antiulcerogenic activity of adiponectin against ethanol induced gastric mucosal injury in rats.
- Investigation of the effects of a combination of metformin and adiponectin on *in vivo* levels of blood glucose and blood lipids in mice.

## **Chapter 2**

# **Literature Review**

## 2.1 The origin of Adiponectin protein

With completion of the human genome project, it has been determined that the molecular mechanism of cell behaviour is not able to be predicted only based on the sequence of the gene. Proteins are responsible for cell behaviour and all activities that are done in the cell. Therefore, in order to identify the molecular mechanism of cellular behaviour and biological reaction, the investigation of proteins which are expressed in a cell, their changes in different conditions, their performance and interaction between different proteins in a cell, are necessary. Thus, the study of proteins and their function is central to understanding both cells and organisms (Chen *et al.*, 1998).

On the other hand, recent studies of adipose tissue have proved that fat tissue is an important and active organ which secretes different hormones to regulate energy metabolism (Kershaw and Flier, 2004). Adipose tissue is considered to be an active endocrine organ, sensing metabolic signals and secreting hormones that affect whole body energy homeostasis. According to a recent research, a numbers of various bioactive proteins known as ‘adipocytokines’ are secreted from adipose tissue (Steppan *et al.*, 2001). These bioactive proteins include adiponectin which has a function in fat cells, tumour necrosis factor- $\alpha$  a mediator of insulin resistance in animal models of type 2 diabetes plasminogen activator inhibitor type 1 (PAI-1) and resistin, they may contribute to the pathogenesis of state coupled with obesity (Spiegelman *et al.*, 1983; Matsuzawa, 2005).

A novel protein which is also synthesized in adipose tissue was described by Philipp E. Scherer, 1995. Human adiponectin is a 244 amino acid protein and it accounts for 0.01% of total plasma protein (Hotta *et al.*, 2000).

Mouse adiponectin is a 247 poly peptide containing a collagen domain at N-terminal and globular C1q-like domain at C-terminal. The N terminus contains a hypervariable region, which is commonly used as the antigenic site for species-specific antibody generation (Pajvani *et al.*, 2003; Wang and Scherer, 2008).

Head of adiponectin globular is formed by C1q-like part, and shares a similar structure with component C1q (Hara *et al.*, 2002), which plays important roles in the innate humoral immune system (Medzhitov and Janeway, 2000; Yamamoto *et al.*, 2005). Although there is no homology at the primary sequence, three dimensional structure is noticeably similar to that of tumour necrosis factor  $\alpha$  (Shapiro and Scherer, 1998). Three adiponectin molecules, which are similar to collagen, can form coiled ring structures very alike to collagen by interaction (Pajvani *et al.*, 2003) and they are essential for the high order multimerization of this protein (Scherer *et al.*, 1995). A 3-30 $\mu$ g/ml is the approximate concentration of adiponectin in the blood, whereas C1q concentration is about 80-200 $\mu$ g/ml and it is interesting to note that although the C1q is the neighbouring structural homolog of adiponectin, anti-adiponectin antibody does not cross react with human C1q (Wouters *et al.*, 2008). A number of oligomeric forms of adiponectin have been described in blood and it is believed that they are separated in the bloodstream and do not convert to each other (Wang *et al.*, 2002). These oligomers are able to bind with  $\text{Ca}^{2+}$  ions and are considered to be involved in the constancy of adiponectin. Trimers, or low molecular weight forms (LMW), are formed by three monomers of this protein, and hexamers and/or medium molecular weight forms (MMW) are formed by linking disulfide bonds between trimers and multimers and/or high molecular weight forms (HMW12 to 36mer) of which the accurate structure is not yet known (Pischon *et al.*, 2004). However, it is believed that a modified amino acid residue in a collagen domain with the contribution of disulfide and some other bounds is captured in proper holding subunits of high molecular weight formed together (Koenig *et al.*, 2006).

### 2.1.1 Adiponectin role

High molecular weight form mediates the biological activity of adiponectin and the correlation of ratio HMW : total adiponectin is stronger than the correlation between the total adiponectin with insulin resistance and other measures of type 2 diabetes (Okamoto *et al.*, 2006). HMW ratio is decreased in obese patients and obese mouse models (Wang and Scherer, 2008). Although adiponectin is derived only from adipose tissue (Scherer *et al.*, 1995; Lindsay *et al.*, 2002), studies of adiponectin in humans have shown an inverse relation between serum concentrations of adiponectin and insulin sensitivity in both non-obese and obese subjects (Orio Jr *et al.*, 2003; Pellme *et al.*, 2003), and also in patients with type 2 diabetes and coronary artery disease (Hotta *et al.*, 2000).

Studies have demonstrated this reverse connection, between reduced levels of adiponectin and the development of insulin resistance and type 2 diabetes (Hara *et al.*, 2002; Graber *et al.*, 2009). Lindsay *et al* showed that type 2 diabetes is more likely to be developed by individuals in the Pima Indian population who had lower serum levels of adiponectin, compared with those with higher levels (Lindsay *et al.*, 2002) Adiponectin levels are negatively correlated with percent of body fat, central fat distribution, fasting plasma insulin, and oral glucose tolerance, however it positively correlated with glucose disposal during euglycemic insulin clamp (Goldstein and Scalia, 2004). A reduction of triglyceride storage in liver and muscle, plasma triglyceride and free fatty acid concentrations and improved hyperglycaemia are the result of long term administration of adiponectin (Yamauchi *et al.*, 2001; Hoffstedt *et al.*, 2004).

Moreover, insulin sensitivity is significantly improved in the treatment of diabetic animals with adiponectin, and this improvement was apparently achieved by reducing triglyceride accumulation which is a result of supporting the oxidation of lipids



(Vionnet *et al.*, 2000; Hara *et al.*, 2002). For example, the suppression of gluconeogenesis by insulin is improved by adiponectin in mouse hepatocytes. Also, insulin resistance and formation of neointimal were observed in adiponectin-deficient mice (Berg *et al.*, 2001; Kubota *et al.*, 2002).

In addition to all recent case researches, there have been a number of other roles of adiponectin proven, such as its effects on weight loss and other useful roles that are briefly described below.

Adiponectin acts in the brain to reduce body weight by raising the metabolic rate, while not affecting appetite. Leptin causes weight loss by suppressing appetite and increasing metabolic rate, but adiponectin can cause weight loss without affecting intake. To compare, both leptin and adiponectin are produced in fat tissue, and both lead to increased metabolic rate. Leptin is known to be a satiety signal that also acts on the brain to decrease appetite (Matsuzawa, 2005).

Other researchers have discovered the role of adiponectin in kidney disease. Scientists realized that mice with low levels of adiponectin have high levels of albumin. Since albumin is an index for renal and kidney disease, this can address the assumption that adiponectin deficiency can be a cause of kidney disease (Stenvinkel *et al.*, 2004; Menon *et al.*, 2006).

These observations recommend that adiponectin plays an important role in the human body (Hara *et al.*, 2002). Although the exact mechanism of action, receptors and signalling pathway of adiponectin have not been completely understood, useful roles in metabolism such as improved insulin sensitivity, glucose tolerance and lipid profile, decreased inflammation, atherosclerosis and a risk of kidney disease are considerable (Capeau, 2007).

### **2.1.2 Adiponectin gene characteristics**

Adiponectin protein is expressed entirely in adipose tissue by the adiponectin gene AMP1 (also known as *GBP28*), which consists of three exons and two introns spanning a 17kb region and it is considered to be a plentiful gene product in adipose tissue. The adiponectin gene is positioned on chromosome 3q27 (Stumvoll *et al.*, 2002; Kang *et al.*, 2005).

Hara 2004 detected a number of single nucleotide polymorphisms (SNPs) located within the adiponectin gene in a Japanese cohort of patients. Kazuo found 85 common nucleotide changes in the adiponectin gene, SNPs 45, 276 (Schulze *et al.*, 2004; Yamamoto *et al.*, 2005) and 1137 that are shown to be associated with type 2 diabetes, circulating adiponectin levels, and insulin resistance in the Japanese population. Significant associations have been detected between SNPs at positions 4034, 3964 and the insulin resistance index as well. However, the result differs according to racial population study (Hara *et al.*, 2002, Kang *et al.*, 2005).

In the adiponectin gene of non-diabetic populations, two uncommon and one common genetic polymorphisms have been identified (Schaffler *et al.*, 2000; Takahashi *et al.*, 2000). A silent T-G exchange in nucleotide 94 (exon 2) (frequency 25%), a T-C exchange in nucleotide 331 (exon 3) (Tyr111His; frequency in Germans 4%), and a T-C exchange in nucleotide 383 (exon 3) resulting in a missense mutation (Arg112Cys; frequency in Japanese 0.5%). Frequency of the polymorphisms was 50% (exon 2) and 0.5% (exon 3). The highly prevalent T-G polymorphism in exon 2, though not resulting in an amino acid exchange, could somehow affect plasma adiponectin levels (Stumvoll *et al.*, 2002). Although it did not reach statistical significance, there was a dose-dependent decrease in the mean adiponectin concentration for the G allele in 219 Japanese (Diez and Iglesias, 2003). Adiponectin receptors have been identified ten years after the discovery of adiponectin in Kadowaki's laboratory (Capeau, 2007).

Related receptors have been isolated by Yamauchi *et al.* from human skeletal muscle by expression cloning known as AdipoR1 and AdipoR2 (Yamauchi *et al.*, 2001). Different genes express these two receptors but 67% of their sequences are in common. The expression of AdipoR1 occurs more in muscle and liver representing the existence of AdipoR2. Implication of these two receptors as crucial mediators for adiponectin signalling has been proven by both *in vitro* and *in vivo* experiments (Wang and Scherer, 2008).

## **2.2 Production of adiponectin**

A number of adiponectin complexes such as (LMW), (MMW) and (HMW), globular domain (gAdiponectin) and full-length adiponectin are present in serum (Pajvani *et al.*, 2003; DeGuo *et al.*, 2007). Hormone concentration is normally below or within the ng/ml, whereas adiponectin concentration varies between 2 to 18µg/ml in plasma. Hence, adiponectin cannot be considered a typical hormone because of its concentration which is not within a hormonal range in plasma (Graber *et al.*, 2009). The “globular head” of adiponectin (gAdiponectin) polypeptides correlate as trimers and in a lower dose are more effective than full-length adiponectin (DeGuo *et al.*, 2007). Serum levels of adiponectin are decreased in several models of obese humans, insulin resistant rodent models, leptin-deficient and leptin-receptor deficient mice, and monkey models (Yamauchi *et al.*, 2001; Yamamoto *et al.*, 2005).

Recently, bioactive adiponectin polypeptides have been studied by different researchers via bacteria recombinant technologies, human cell lines, insect cell lines and plants (Fruebis *et al.*, 2001; Neumeier *et al.*, 2006). However, obtaining only small amounts of adiponectin was expressed in almost all related publications. In addition, production of adiponectin by mammalian cell lines, which includes the manipulation and culture of

mammalian expression system, is difficult and costly (Arita *et al.*, 1999; Berg *et al.*, 2001). The purification of a fusion protein comprised of an adiponectin polypeptide and a tag such as His-Tag, have been disclosed in most of these publications. Expression of mouse adiponectin in plants was first established by (Berberich *et al.*, 2005). Transgenic sweet potato plants were used for developing mouse adiponectin cDNA via *Agrobacterium*-mediated transformation. Since plants use only water, carbon dioxide, sunlight and minerals as energy sources, plant-based expression of pharmaceutical proteins such as adiponectin is cost-effective in comparison to other production systems mentioned above. Plants also present a suitable modification system, such as folding and post-translational modification for eukaryotic proteins. Using the plant-based expression system is also safer because it avoids animal cell-culture contaminants such as mammalian pathogens and extraneous viral or bacterial components (Ma *et al.*, 2003; Peterson and Arntzen, 2004). Although the advantages of producing adiponectin from plants are considerable (Fruebis *et al.*, 2001; Liu *et al.*, 2006) the purity is only 90% and the yield of production is low. Hence, there is a necessity for a process obtaining high yield of active and pure adiponectin polypeptides and purification process of adiponectin polypeptides (Graber *et al.*, 2009).

A variety of heterologous proteins has been produced via a successful expression system which is methylotrophic yeast *Pichia Pastoris* (Macauley-Patrick *et al.*, 2005). *P. pastoris* has many advantages of the higher eukaryotic expression system such as protein processing, protein folding and post-translational modification. Its expression system is a unique system for the production of high levels of recombinant proteins. These features and flexibility of the *P. pastoris* expression system, make it an ideal tool for laboratory research, as well as industrial application.

High-level expression of human gAdiponectin in *P. pastoris* has been reported by and a pilot procedure had been established to produce the recombinant gAdiponectin. The

recombinant gAdiponectin has been proven to be fully functional in both cell culture models and animal models (Graber *et al.*, 2009).

### **2.3 Diabetes and obesity**

Among two different types of diabetes, type 2 diabetes accounted for 90-95% of all cases. This heterogeneous disorder afflicts an estimated 6% of the adult population in Western society. Diabetes is not only considered the fifth leading cause of death in the U.S. and the sixth in the world, but it is also considered to be a precondition for heart disease, blindness, kidney failure, and other chronic conditions (Mokdad *et al.*, 2003).

The number of patients with diabetes was estimated to be 135 million in 1995, 154 million people in 2000, 200–300 million cases in 2010 and it is likely to reach above 300 million by 2025 around the world (Yang *et al.*, 2001). The latest studies which was estimated in 2000 one in three born Americans would develop diabetes in their life and all developed nations will confront this threat soon (Moller, 2001; Diez and Iglesias, 2003). The diabetes epidemic is followed by high cost in dollars, health complications, mortality and morbidity (Hogan *et al.*, 2003, Lazar, 2005).

Type 2 diabetes mellitus (T2D), which in earlier times was called non insulin dependent diabetes, is a metabolic disorder that is noticeable with increased levels of plasma glucose ahead of the normal physiological range and is characterized by a progressive loss of glycemic control (Winter *et al.*, 2006). The main feature of almost all patients with type 2 diabetes is insulin resistance (Tonelli *et al.*, 2004). It is interesting to note that the reason for this common disorder is closely associated with increased body weight, sedentary lifestyle and family history of diabetes (Goldstein and Scalia, 2004). According to a study by Moller, 2001, the main force driving this increasing incidence is also a staggering increase in obesity, the single most important

contributor to the pathogenesis of diabetes. Obesity is associated with a high fat (HF) diet and this circumstance subsequently induces insulin resistance which is a major risk factor for diabetes and cardiovascular diseases (Kadowaki and Yamauchi, 2005).

During periods of prolonged famine that plagued early human hunter-gatherers, a survival advantage would have been conferred by genes that favour the economical use and storage of energy: the so-called “thrifty” gene (Weiss and Ward, 2000). The so-called thrifty genes, were used as energy storage during periods of famine. Our biological system stores fat as energy by thrifty genes, therefore supporting a raise in adipose tissue is considered to be another function of the thrifty genes (Weiss and Ward, 2000; Lazar, 2005). Since glucose is used as biological fuel, the reason for genetic tendency toward insulin resistance is the attempt and pressure to conserve glucose during starvation for use by the brain (Lazar, 2005). Thrifty genes have been suggested to be a cause of diabetes and obesity in a sedentary lifestyle. Since obesity has a clear genetic element and is not often monogenic (O’Rahilly *et al.*, 2003), multiple thrifty genes are more likely to be. Susceptibility to obesity and diabetes varies depending on the inheritance of a number of polymorphisms which can lead to tiny differences in expression (Diamond, 2003).

### **2.3.1 Adiponectin and diabetes**

Body fat deposition or insulin resistance can be promoted by separate sets of thrifty genes, and in fact, production and storage of fatty acid is increased by insulin in adipose tissue, thus aggravating obesity. Interestingly, adipose tissue is recognized to

correspond with peripheral tissues and the brain by regulating appetite and metabolism through secreting hormones (Kershaw and Flier, 2004). The concentration of adiponectin in plasma in those who later develop type 2 diabetes as reported by Spranger *et al.*, 2003 was lower than in control groups (mean 5.34g/mL [SD 3.49] vs 6.87g/mL [4.58],  $p < 0.0001$ ), hence adiponectin concentrations in plasma are independently associated with a risk of type 2 diabetes. After correction and adjustment for sex, BMI, age, smoking, exercise, alcohol consumption, waist-to-hip ratio, education, and glycosylated haemoglobin, high concentrations of adiponectin were associated with a significantly reduced relative risk of type 2 diabetes (Spranger *et al.*, 2003).

Adiponectin, especially HMW adiponectin which is a more active form of this protein, has an important role in insulin sensitivity and in protecting against diabetes. A number of reasons support this hypothesis (Kadowaki *et al.*, 2006). First, rare mutations like SNPs 1137 are closely associated with type 2 diabetes. Subjects with either of these mutations have extremely low levels of HMW adiponectin. In addition, the mutant type of adiponectin expressed in NIH-3T3 fibroblasts was not capable of forming the HMW form of adiponectin (Hara *et al.*, 2002; Waki *et al.*, 2003). Second, in both mice and human diabetic patients an increase in the ratio HMW-to-total adiponectin, levels correlate with improvement in insulin sensitivity (Pajvani *et al.*, 2004). This correlation suggests that alternation in HMW plasma adiponectin levels to total plasma adiponectin may be more relevant and have significantly better power for the prediction of insulin resistance and the metabolic syndrome in humans (Ebinuma *et al.*, 2006; Kadowaki *et al.*, 2006).

Subsequently, levels of the ratio of total adiponectin, LMW adiponectin and HMW adiponectin and HMW-to-total adiponectin are all considerably correlated with the main characteristics of central obesity and the disposal rate of insulin-stimulated glucose.

However, HMW adiponectin level may be the greater biomarker for insulin resistance, metabolic syndrome, and type 2 diabetes (Kadowaki *et al.*, 2006).

#### **2.4 Drugs for diabetes treatment**

Treatment of type 2 diabetes mainly relies on a number of approaches nowadays. Most of these treatments such as sulphonylureas and thiazolidinediones are planned to decrease hyperglycaemia. A number of available therapies is effective in addressing some weaknesses such as obesity and/or insulin resistance (Drucker, 2001).

Sulphonylureas (and related insulin secretagogues) increases insulin release from pancreatic islets, but a matter with sulphonylureas in patients leads them to become refractory to treatment over time. Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) agonists (thiazolidinediones) improve insulin action,  $\alpha$ -glucosidase inhibitors interfere with gut glucose absorption and insulin itself suppresses glucose production and augments glucose utilization (Drucker, 2001, Moller, 2001). One of the major concerns is the tendency to gain weight in almost all treatments.

However, new approaches are seriously required. Accurate focus has to be placed on discovering new mechanisms of drugs, which must depend on physiological responses that affect weight loss. Several aims should be taken into account for new therapeutic approaches such as reducing glucose production by liver, enhancing glucose-stimulated insulin secretion, reducing fat and changing lipid metabolism (Zhang and Moller, 2000).

##### **2.4.1 Metformin**



Metformin, which acts to reduce hepatic glucose production, has been reported to reduce or at least keep weight constant. Metformin belongs to a class of oral medicine (dimethylbiguanide) used to treat Type 2 diabetes, and is known as biguanide. The use of this drug has been accepted by the Food and Drug Administration in the United States. The usage of metformin as monotherapy is as successful as insulin and sulfonylureas, and does not affect body mass index (BMI) like sulfonylureas, insulin, and thiazolidinediones (Kirpichnikov *et al.*, 2002; Saaddine *et al.*, 2002).

Thus, it is considered to have exceptional benefits in obese patients with type 2 diabetes. The efficiency of metformin monotherapy does not depend on age, duration of diabetes, body weight and insulin level. It also has been reported that metformin is not only effective when it is used as monotherapy, but also when it takes part in combination with insulin, sulfonylureas, and thiazolidinediones. Hence, the hypothesis of effectiveness of a combination of metformin with adiponectin is one of the main objectives of the present study (Reaven *et al.*, 1992; Levri *et al.*, 2005).

Reduction in glucose concentration by 2.78 to 3.90mmol/L (50 to 70mg/dL) is observed in the combination of metformin with diet (Kirpichnikov *et al.*, 2002). Metformin represents cardiovascular protection in diabetic patients that seems to be related with the action of this drug on metabolism of lipids, vascular smooth-muscle and cardiomyocyte intracellular calcium handling, endothelial function, hypercoagulation, and platelet hyperactivity. Losing weight in diabetic patients and patients with polycystic ovary syndrome (PCOS) is the additional feature of using metformin (Bailey and Turner, 1996; Levri *et al.*, 2005). Considerable reduction in total body fat and visceral fat in women with pre-existing abdominal obesity has been observed after using metformin. Decreased net caloric intake most likely through appetite repression during metformin treatment leads to weight loss and this effect is totally free of gastrointestinal side effects of metformin. In cardiovascular metabolic syndrome fat localized on the

paraintestinal region and reduction in visceral fat may have additional cardiovascular benefits in insulin-resistant persons treated with metformin (Palumbo, 1998; Bailey, 2008). A complete oral bioavailability of 50% to 60% has been achieved at doses of 500 to 1500 mg. Inside the body, metformin is not modified and the kidneys secrete it through glomerular filtration (tubular secretion). Accumulation of metformin may occur due to impaired kidney function (Kirpichnikov *et al.*, 2002; Huypens *et al.*, 2005). Decreased hepatic glucose production and concentration of fasting plasma glucose by 25% to 30% by raising the binding of insulin to its receptors and translocation of GLUT-1 and GLUT-4 glucose transporters in certain tissues, stimulates catabolic pathways, and consequently reduces adipocyte size which are the main actions of metformin (Larkins, 1997; Ciaraldi *et al.*, 2002).

#### **2.4.2 Mechanism of metformin**

Reduction of hepatic glucose production, which is increased at least twofold in patients with type 2 diabetes, and increased insulin-stimulated glucose uptake in adipocytes and skeletal muscle, are the mechanisms of antihyperglycemic action of metformin. Reduction of gluconeogenesis mostly through inhibition of hepatic lactate uptake in the liver was observed with use of metformin (DeGuo *et al.*, 2007). It is interesting to know that a reduction in concentration of adenosine triphosphate in isolated rats, hepatocytes have been reported as another function of metformin. Since adenosine triphosphate is an allosteric inhibitor of pyruvate kinase, the metformin-mediated lessening in hepatic glucose formation results from enlarged pyruvate kinase fluctuation (Bailey and Turner, 1996; Kirpichnikov *et al.*, 2002). Metformin also decreases gluconeogenic fluctuation through inhibition of pyruvate carboxylase–phosphoenolpyruvate carboxykinase activity and perhaps through increased transfer of pyruvate to alanine. In addition,

metformin facilitates insulin induced suppression of gluconeogenesis from a number of substances, counting glycerol, lactate, amino acid and pyruvate, and opposes the gluconeogenic actions of glucagon. In skeletal muscles and a number of other tissues, trafficking of glucose transporters is facilitated by metformin to the plasma membrane. The capacity of glucose transporters may also be increased by metformin. The presence of insulin in peripheral insulin-sensitive tissues is required for full action of metformin. The majority of biological activities such as glycogen and glucose transport and lipid synthesis of insulin are enhanced by metformin (Levri *et al.*, 2005; Huypens *et al.*, 2005). In the lack of insulin in cultured skeletal muscle, metformin facilitates glucose transport. In vascular smooth-muscle cells, tyrosine kinase action and insulin in insulin-like growth factor-1 receptor are made active by metformin independently of insulin action. Tyrosine kinase in *Xenopus* oocytes is activated by this drug with following stimulation of inositol 1,4,5 triphosphate creation and glycogen production. Hence, this drug has metabolic properties on insulin-sensitive tissues that may possibly contribute to its glucose-lowering outcome (Bailey and Turner, 1996; Winter *et al.*, 2006). In diabetes and obesity, high levels of free fatty acid are frequently seen. Elevated levels may widen the insulin resistance and possibly contribute to increase hepatic glucose production. Lessening in oxidation of free fatty acid by 10% to 30% has been achieved by metformin. Acetyl coenzyme A and citrate are by-products of free fatty acid oxidation. Accumulation of these by-products happens when fatty acid oxidation increases stalls and inhibits key enzymes of the glycolytic pathway. Metformin can enhance insulin sensitivity and also assist in the proper secretion of insulin by  $\beta$ -cells, by decreasing free fatty acid levels. Although there is no direct effect of metformin on  $\beta$ -cell function, insulin secretion that has been changed by long-standing exposure to free fatty acid or hyperglycaemia (glucose toxicity) can be improved. In addition, a decline in absorption of glucose through the intestine may also be achieved by

metformin, as it improves hyperglycaemia by attaining high concentrations in the small intestine. Glucose transport to the hepatic circulation may be prevented in metformin treated patients by increased glucose consumption in the small intestine (Moggetti *et al.*, 2000; Patane *et al.*, 2000).

In summary, a decline in hepatic glucose production by inhibiting gluconeogenesis as well as enhanced peripheral insulin sensitivity, have been achieved by metformin. Furthermore, the decrease in gastrointestinal glucose absorption and reduced glucose toxicity and free fatty acid levels which lead to improved pancreatic  $\beta$ -cell response to glucose, are indirect roles of metformin.

#### **2.5 Adiponectin and anti-inflammation activity**

Obesity changes various immunologic functions, since the immune system in humans is linked with adipose processes. Obese people have an impaired sensitivity of lymphocytes, with a decreased B and T-cell function. Serum levels of TNF- $\alpha$ , IL-6, and CRP are considerably high in obese people, thus they are expected to die independent of age, sex, BMI, or diabetes because of cardiovascular and non-cardiovascular causes (Das, 2004)

As mentioned earlier, adiponectin is confirmed to be associated with avoidance of atherosclerosis, decreased inflammation, prevention of fatty liver and liver fibrosis and improved insulin sensitivity, glucose tolerance and lipid profile. About the anti-inflammatory effects of adiponectin, expression of vascular cell adhesion molecules is inhibited by adiponectin, thus, monocyte connection to cultured human aortic endothelial cells is reduced (Ouchi *et al.*, 2000) . Inhibition of phagocytic activity, production of pro-inflammatory cytokines such as TNF- $\alpha$  in human macrophages, and moreover, enhancement in the production of IL-10 and tissue inhibitor of

metalloproteinase 1 in human cultured macrophages that have been reported by (Yamamoto *et al.*, 2005), are considered to exhibit anti-inflammatory properties of adiponectin particularly in endothelial cells and macrophage activity. Interestingly, IL-4, IL-10, and TGF- $\alpha$  have anti-inflammatory action and inhibit the production of pro-inflammatory cytokines IL-1, IL-2, and TNF- $\alpha$  (Das, 2004). Adiponectin induces the production of these anti-inflammatory agents by exerting immuno-suppressive properties (Wolf *et al.*, 2004).

Another anti-inflammatory activity of adiponectin is anti-atherosclerosis. The risk factors including obesity, diabetes, high blood pressure, family history of heart disease, high cholesterol or simply growing older, all affect our arteries. Different types of cholesterol flow inside arteries. The most important type is LDL cholesterol, known as bad cholesterol because it can lead to clogged arteries. HDL cholesterol is called good cholesterol because it is believed to help remove bad cholesterol from the body. When there is too much LDL in the blood, this can cause serious problems. When there is damage to the lining of the arteries due to high blood pressure, LDL cholesterol is more likely to enter the artery walls. When LDL enters the damaged wall of the artery, it could lead to inflammation (Ouchi *et al.*, 2001; Okamoto *et al.*, 2002). Over time, inflammation and the artery wall create something known as plaque. Plaque could start forming at an early age and over time this can lead to clogging of the arteries. This can happen at a number of different sites around the body and can get progressively worse over time. This chronic process known as atherosclerosis can become life threatening depending on whether the plaque is stable or unstable. Stable plaque can continue to grow slowly reducing blood flow over time by hardening and narrowing the artery, but does not necessarily completely block the flow of blood (Ekmekci and Ekmekci, 2006). Sometimes, even small plaque could become unstable and explode its contents. This is much more dangerous than stable plaque because a clog could quickly form and

completely block the flow of blood. As stable plaque forms causing hardening and narrowing of the leg arteries, the blood flow is restricted and causes pain. Elsewhere in the body the effects of this can be immediate. A blockage of arteries supplying blood to the heart can cause a heart attack. A blockage of arteries supplying blood to the brain can cause a stroke (Ouchi *et al.*, 2000; Yamamoto *et al.*, 2005).

Since cholesterol deposits in arteries of patients with type 2 diabetes are much higher, an increase in adiponectin levels might be a helpful target for decreasing the atherosclerotic risk present in diabetes (Schulze *et al.*, 2004).

Accumulation of adiponectin in the subintimal space of the arterial wall and its interaction with collagens in the vascular intima when the vascular endothelium is injured, have indicated that adiponectin has potential anti-atherogenic properties (Ouchi *et al.*, 2001; Ekmekci and Ekmekci, 2006).

Immunohistochemical studies also exposed the adhesion of adiponectin from the plasma to the injured artery. Human recombinant adiponectin suppressed the endothelial expression of adhesion molecules, the transformation of macrophage to foam cells and the proliferation of vascular smooth muscle cells in culture cells. This data also suggests that adiponectin has anti-atherogenic properties (Ouchi *et al.*, 2003).

Furthermore the function of adiponectin in the pathogenesis of Crohn's disease (CD) also reported by Yamamoto *et al.*, 2005. CD is a chronic inflammatory bowel disease of the gastrointestinal tract. Identification and diagnosis of CD are done by a set of histological, endoscopic, macroscopic and clinical characteristic evaluations (Yamamoto *et al.*, 2005).

## **2.6 Gastric mucosal injury**

In addition to this case, it is also worth noting that one of the more common disorders in communities nowadays is gastric ulcer. Certain types of substances including drugs often have an effect on the stomach and cause indigestion or damage to the mucosa. The most common chemical which is used by multiple people is alcohol.

Since ethanol is a simple method used for inducing gastric ulcer in experimental rats and leads to severe gastric mucosal injury, wide research has been established to study the effects of alcohol on human gastric mucosa, and high concentrations of ethanol to influence the complexity of deep influence vascular stasis, impaired blood vessel walls and necrosis of deep mucosa. It has been reported by Hattori *et al.*, 2008 that subsequent to congestion of the gastric mucosal microcirculation, the cause of gastric mucosal disclosure to ethanol and an important cause of injury is disturbance in the gastric mucosal microcirculation and destruction of the gastric mucosal barrier consisting of the surface epithelium and mucosal coat. Regarding anti-inflammatory properties of adiponectin, the effect of this protein on mucosal injury might be noticeable. Activity of adiponectin against ethanol-induced gastric mucosal injury has not been examined clearly up to now. Hence, the study herein was designed to evaluate the anti-ulcerogenic activity of adiponectin against ethanol-induced gastric mucosal injury in rats (Ohno *et al.*, 1995; Hattori *et al.*, 2008).

## **Chapter 3**

# **Materials and Methodology**



### **3.1 Recombinant yeast**

Recombinant yeast was obtained from the adiponectin expression project which had been previously carried out. Plasmid DNA was first linearized by restriction enzyme *Sac I*, (Fermentas, Canada) and then transformed in to *P. pastoris* stain X33 (Prondisa, Canada) by using EasyComp transformation kit (Invitrogen, U.S.A), following manufacture instruction.

### **3.2 Growth media preparation for protein expression**

Recombinant yeast was cultured in order to produce adiponectin. First, Buffered Glycerol-complex Medium (BMGY) media was used as a growth media having glycerol as carbon source. The media was prepared (as shown in the Appendix), in a 100ml Erlenmeyer flask and autoclaved for 15min at 121°C. A single recombinant *P. pastoris* colony was inoculated into a 50ml BMGY medium and grown in a 100ml Erlenmeyer flasks at 30°C in a shaker incubator at 230g, overnight. Cells were harvested by centrifugation for 8min at 3000g and pellets were mixed with Buffered Methanol-complex Medium (BMMY) media. (Medium was supplemented with 5ml of methanol instead of glycerol). Since the base of the BMMY is similar to BMGY, it was prepared and autoclaved during the preparation of BMMY media, only methanol was added to the base of BMMY media and pellets were mixed with it.

The media was allowed to grow for 48hr in 250ml Erlenmeyer flasks at 30°C with the same shaker parameters. Every 12h, 0.5ml methanol was added to the culture to induce growth to maximum level. In order to separate the protein from the yeast, the culture medium was centrifuged for 8min at 3000g after a certain time (48hr) and the supernatant was stored separately overnight at 20°C. The supernatant was poured into a

plastic bottle and 3 volumes of acetone 150ml were added in order to precipitate the protein, and then kept at 20°C overnight. Bottles were centrifuged for 30min at 4°C and 1000g. Acetone was removed and pellets were mixed with 5ml PBS potassium phosphate saline buffer which helped to separate soluble protein from insoluble protein. The solution was transferred to 1.5ml tubes and centrifuged again for 5min. The supernatant that contained the soluble protein was collected and subjected to purification process.

### **3.3 Purification of adiponectin**

Purification was done by nickel-based purification columns in order to achieve a sufficient quantity of purified adiponectin for subsequent use. Sodium phosphate buffer was applied to the columns and the process was followed by applying the supernatant to nickel-based purification columns, which is shown in the Appendix. After that, binding buffer was applied to the columns and proteins containing His tags were bound to the nickel, whereas non-specific proteins were eluted from the column. The His tagged proteins were eluted with elution buffer that contained imidazole which displaced the His from the nickel. The eluant was collected into tubes.

## **3.4 Protein Study**

### **3.4.1 SDS –PAGE**

In order to confirm the presence and estimated size of the adiponectin protein SDS-PAGE was performed. An 8ml of acrylamide solution was prepared in 1.0mm thickness. The preparation of acrylamide solution for 12.5% homogenous gels is described in the appendix. The apparatus was set up and the appropriate volume of APS and TEMED was added to the gel solution and stirred only when ready to pour the gel

solution into the caster. The above homogenous solution was poured reaching 1.5-2cm from the top. Immediately, 1ml of water-saturated n-butanol was slowly pipetted onto the gel to keep the gel from drying out. After 30min the water-saturated n-butanol was removed by tissue, and the stacking solution was poured and combs were placed onto the gel in order to make wells. Care was taken to avoid air bubble accumulation. The gel was left for 30min to polymerize.

The samples were spin for 5min and 18ml marker (mercaptol ethanol) and 2ml of loading dye were added to 20ml of each sample. Samples were heated at 100°C for 6min for protein denaturation purpose. After 30min, when the gel was ready, a gel cassette sandwich was removed from the casting frame, placed into the electrode system assembled into the clamping frame system, and poured with 1X running buffer electrophoresis until the buffer covered the gel. The combs were removed by pulling straight up carefully. 20 ml of protein ladder (Promega, U.S.A) and samples were loaded and the gel was run at 180 vol. for 60min in a running buffer. After that the gel was removed from the glass plate and placed in a plastic container. The gel was covered with fixing solution and shaken on the shaker for 30min at room temperature. The fixing solution was poured off and an infiltrating solution was poured on the gel and shaken at the same condition. Then, the gel was washed with dH<sub>2</sub>O three times for 5min and then was shaken on the shaker. A silver solution was poured onto the gel after washing and left on the shaker for 20min. A staining solution was poured onto the gel and the plastic container was shaken gently by hand until bands appeared. Once the bands appeared, the staining solution was poured off to prevent the gel from darkening. The membranes were visualized using an enhanced chemiluminescence detection system.

### **3.4.2. Western blotting**

The glass plates were assembled vertically according to the instruction of the manufacturer. The resolving gel was prepared in 1.0M Tris-HCl, pH 6.8. Gel contained 0.1% (w/v) SDS and was polymerized with 0.1% (w/v) APS and 0.1% (v/v) TEMED. The gel was polymerized, with the resolving gel at the bottom and the stacking gel on the top where the wells were formed. After the polyacrylamide gel had polymerized, the gel sandwich was secured to a vertical electrophoresis apparatus. The upper and lower buffer chambers were filled with SDS-PAGE buffer until the top and bottom ends of the gel sandwich were submerged in the buffer. Before the protein samples were loaded, the wells were flushed with SDS-PAGE buffer to remove any small pieces of gel left in the wells. Heat-treated samples, 10 $\mu$ l each, with pre-stained marker, were loaded into the wells. Electrophoresis was performed at a constant voltage of 200V until the bromophenol blue reached the bottom of the gels. Gel-membrane was assembled into the sandwich clamp according to the instructions of the manufacturer. The membrane was soaked in transfer buffer for 10min and transferred to a nylon membrane. Then the membrane was soaked in blocking buffer at room temperature for one hour. The membrane was swirled in probing solution with 5 $\mu$ l of primary anti-body (anti His antibody) for 30-60min at room temperature. The membrane was rinsed with 1X TBS after swirling, and was followed by washing the membrane with 1X TBST, 3 times, for 5min and again rinsed with 1X TBS. Next, the membrane was swirled in probing solution with 2 $\mu$ l of a secondary antibody (masc-IgG) for one hour at room temperature. After swirling with the secondary antibody the membrane was rinsed with 1X TBS and washed 3 times with 1X TBST for 15min each time. Before adding the substrate, the membrane was rinsed one more time with 1X TBS. Then, 3ml of substrate for alkaline phosphatase TMB western blue was added, and without swirling, the membrane was stored in the dark until bands appeared. Once the bands appeared, the colour

development was stopped by rinsing the membrane with sdH<sub>2</sub>O. The membranes were visualized using an enhanced chemiluminescence detection system.

### **3.5 Mice preparation for Adiponectin-Metformin combination**

Healthy, 18 females ICR mice were used as a model system. Animals were obtained from the Animal House, Faculty of Medicine, University of Malaya in Kuala Lumpur (Ethics No. PM 07/05/2008 MAA (a) (R) to compare the biological activity of the Adiponectin-Metformin combination. Animals, each with mean body weight of 27g were assigned equally into 3 groups (3 groups, n=6 each) labelled as: control, adiponectin and metformin-adiponectin groups. Following overnight fasting, the animals were gavaged with a 1% volume / body weight high fat-sucrose diet (12ml water, 6g sun flower oil, 6g butter, 10g sucrose and 10g skim milk). A dosage of 2.5mg/kg/body weight recombinant adiponectin was injected intraperitoneally while a metformin dosage of 250mg/kg/body weight was given orally by adding to the feed.

Blood glucose was measured with a glucometer (Contour™ Bayar, Hong Kong ) at one hour intervals for 5 hours. Available commercial kits were used to determine blood concentration of triglyceride (TG), total cholesterol (CHOL) (Siemens, USA), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Dade Behring, USA). The difference in blood glucose levels between the 2 groups was assessed by comparing means via the most common type of *t*-test which is "two-sample *t*-test" also known as the "Student's *t*-test" or the "independent samples *t*-test.

### **3.6 Acute toxicity test LD<sub>50</sub>**

#### **3.6.1 Preparation of mice**

Adult BALB cJ male and female healthy mice (6-8 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics No. PM 07/05/2008 MAA (a) (R)). The mice weighed between 30–40g. The animals were given standard rat pellets and tap water ad libitum. The acute toxic study was used to determine a safe dose for the adiponectin. Thirty six mice (18 males and 18 females) were assigned equally into 3 groups labelled as: vehicle (distilled water) group number 1; 2mg/kg and 5mg/kg of adiponectin group number 2 and 3 respectively. The animals were fasted overnight of food but not water, prior to dosing. Food was withheld for a further 3 to 4 hours after dosing. The animals were observed after administration at 30min and 2, 4, 8, 24 and 48h for the onset of clinical or toxicological symptoms. Mortality, if any, was observed over a period of 2 weeks. The acute toxicity LD<sub>50</sub> was calculated as the geometric mean of the dose that resulted in 100% lethality, which caused no lethality at all. The animals were fasted on the 14<sup>th</sup> day and sacrificed on the 15<sup>th</sup>. Histology, haematological and serum biochemical parameters were determined following standard methods (Bergmeyer, 1980; Tietz *et al.*, 1983). The study was approved by the ethics Committee for animal experimentation, Faculty of Medicine, University of Malaya, Kuala Lumpur. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institute of Health.

#### **3.6.2. Behavioural observation and mortality**

Throughout the period of the study, all animals were observed for behavioural signs of toxicity, morbidity and mortality. Mortality checks were made twice daily and

determination of behavioural signs was observed once daily for all animals. Detailed observations of the individual animals were made weekly in comparison with the vehicle treated animals. Observations included gross evaluations of the skin for any sign of respiration (dyspnea), salivation, exophthalmia, convulsion and any changes in locomotion such as whether the animals tend to stay quietly or actively moving in their cage (Rogers *et al.*, 2002).

### **3.6.3 Haematology**

The animals were fasted overnight prior to necropsy and blood was collected. Blood samples were drawn from jugular vein under diethyl ether anaesthesia. Blood samples were collected into EDTA tubes for total and differential white blood cell (WBC) count.

### **3.6.4 Serum biochemistry**

For serum biochemistry analysis, blood was collected into clot-activator tube. Serum biochemistry parameters which were analysed as follows: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total protein, Albumin, Globulin, Total bilirubin, Conjugated bilirubin, Alkaline phosphatase, Gamma glutamyl transferase (GGT), Urea, Creatinine, Anion gap and serum electrolytes (CO<sub>2</sub>, Potassium, Sodium and Chloride). All samples were sent immediately to the Clinical Diagnostic Laboratory at UMMC for liver and renal function tests. Later on, the results were compared to rats' respective control groups.

### **3.6.5 Gross necropsy and histopathology**

At scheduled termination, all surviving animals were anaesthesia by Diethyl ether inhalation and quickly sacrificed by exsanguinations of jugular vein for blood sample collection. Gross post-mortem examinations were performed on all terminated animals. Liver and kidney from each animal were routinely processed and embedded in paraffin. After sectioning and staining with Haematoxylin and Eosin (H&E) stain method, all

slides were observed under microscope with magnification of x10, x40 and x100 (oil immersion) in order to observe for any pathological changes.

### **3.6.6 Statistical analysis**

Data was expressed in mean  $\pm$ S.E.M. Comparisons of body weights, renal and liver function parameters were done against the animals' respective vehicle groups using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test with SPSS Statistics 18.0 software. The treatment groups were significant when *P* value is less than 0.05.

## **3.7 Antiulcer activity**

### **3.7.1. Omeprazole**

Omeprazole is a proton pump inhibitor has been widely used as acid inhibitor agent for the treatment of disorders related to gastric acid secretion for about 15 years (Li *et al.*, 2004). Omeprazole is substituted with benzimidazoles and it inhibits acid secretion by acting on the hydrogen-potassium exchanger ( $H^+K^+$ -ATPase) for the apical plasma membrane of the gastric mucosa (Sato *et al.*, 1989). Omeprazole is highly selective for the proton pump and undergo catalyzed conversion into active form within the acid forming space. The active inhibitors react with SH (thiol) group of the proton pump, resulting in inhibition of acid formation (Nagaya *et al.*, 1991). In this study, omeprazole was used as the reference anti-ulcer drug, and was obtained from the (UMMC) Pharmacy. The drug was dissolved in distilled water and administered orally to the rats in concentrations of 20 mg/kg body (5 ml/kg) (Pedernera *et al.*, 2006).



### **3.7.2 Experimental animals**

*Sprague Dawley* healthy adult male rats were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur, (Ethic No. PM/27/07/2009/MA (A) (R)). The rats were divided randomly into 4 groups of 10 rats each. Each rat that weighted between 180-200 g was placed individually in a separate cage (one rat per cage) with wide-mesh wire bottoms to prevent coprophagia during the experiment. The animals were maintained on a standard pellet and tap water diet. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia. Throughout the experiments, all animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institute of Health.

### **3.7.3 Gastric ulcer induction by ethanol and tissue sample collection**

The rats were fasted for 48 hours before the experiment (Garg *et al.*, 1993), but were allowed free access to drinking water up to 2 hours before the experiment. Gastric ulcer in *Sprague Dawley* rats was induced by orogastric intubation of absolute ethanol (5ml/kg) according to the method described by De Pasquale *et al.*, (1995) with slight modification (De Pasquale *et al.*, 1995). The ulcer control groups were orally administered with vehicle (distilled water, 5ml/kg). The reference group received oral doses of 20mg/kg omeprazole in vehicle (5ml/kg) as positive controls. The experimental groups were orally administered with 2.5ml/kg and 5ml/kg of adiponectin each, respectively. One hour after this pre-treatment, all groups of rats were gavaged with absolute ethanol (5ml/kg) in order to induce gastric ulcers (Hollander *et al.*, 1985). The rats were euthanized by cervical dislocation 60 minutes later (Paiva *et al.*,

1998), under overdose of diethyl ether anaesthesia, and their stomachs were immediately excised.

#### **3.7.4 Measurement of acid in gastric juice**

Each stomach was opened along the greater curvature. Samples of gastric contents were analyzed for hydrogen ion concentration by pH-meter titration with 0.1 N NaOH solutions using a digital pH meter. The acid content was expressed as mEq/l (Andrews *et al.*, 2008).

#### **3.7.5 Measurement of mucus production**

Gastric mucus production was measured in the rats that were subjected to absolute ethanol-induced gastric mucosal injury. The gastric mucosa of each rat was gently scraped using a glass slide and the mucus obtained was weighed using a precision electronic balance (Galati *et al.*, 2002).

#### **3.7.6 Gross gastric lesions evaluation**

Any ulcers would be found in the gastric mucosa, appearing as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Each gastric mucosa was thus examined for damage. The length (mm) and width (mm) of the ulcer on the gastric mucosa were measured by a planimeter ( $10 \times 10 \text{ mm}^2 = \text{ulcer area}$ ) under dissecting microscope (1.8x). The area of each ulcer lesion was measured by counting the number of small squares,  $2 \text{ mm} \times 2 \text{ mm}$ , covering the length and width of each ulcer band. The sum of the areas of all lesions for each stomach was applied in the calculation of the ulcer area (UA) wherein the sum of small squares  $\times 4 \times 1.8 = \text{UA mm}^2$  as described by Kauffman and Grossman (1978) with slight modification. The inhibition percentage (1

%) was calculated by the following formula as described by Njar *et al.*, (1995) with slight modification.

$$(I\%) = [(UA_{\text{control}} - UA_{\text{treated}}) \div UA_{\text{control}}] \times 100.$$

### **3.7.7 Histological evaluation of gastric lesions**

Specimens of the gastric walls from each rat were fixed in 10% buffered formalin and processed in a paraffin tissue processing machine. After embedding sections of the stomach were made at a thickness of 5 $\mu$ m and stained with Haematoxylin and Eosin for histological evaluation.

### **3.7.8 Statistical analysis**

All values were reported as mean  $\pm$  S.E.M. The statistical significance of differences between groups was assessed using one-way ANOVA. A value of  $p < 0.05$  was considered significant.

## **Chapter 4**

# **Result**

#### 4.1- Confirmation of expression of Adiponectin through SDS-PAGE and Western blotting

SDS-PAGE analysis confirmed the expected size of the adiponectin protein 30kDa, (Figure 1a) and Western-blot confirmed the presence of the adiponectin protein. Western blotting showed that the expression band has strong reactivity against monoclonal mouse Anti-His antibody (Fig. 1b), which indicated that recombinant protein has successfully been expressed in *P. pastoris* system.

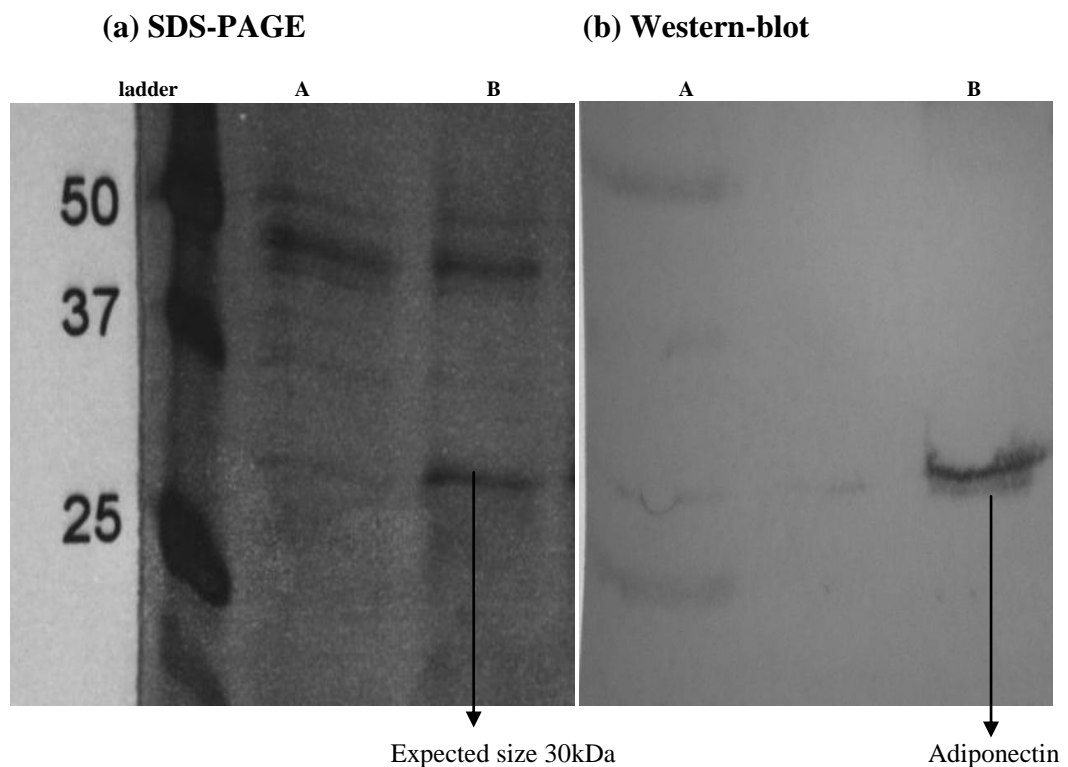


Figure 1 (a, b) A band at 25-37 kDa labelled B, shows the size of Adiponectin which is confirmed by SDS-PAGE and Western blotting.

#### 4.2- Elaboration of the Glucose level

According to *t*-test statistical analysis, difference of glucose level in blood between the metformin group and control group was significant (*p* value <0.01) at 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> hours (*p* value <0.05) and there is no significance difference (*p* value > 0.05) at 4<sup>th</sup> and 5<sup>th</sup> hours after feeding and administration of metformin. The difference between met-adiponectin group and control group was significant (*p* value <0.01) at every hours of the experiment (table 1). Figure 2 shows that the difference between metformin group and metformin-adiponectin group was not significant (*p* value > 0.05) at 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> hours but it is significant (*p* value <0.05) at 4<sup>th</sup> and 5<sup>th</sup> hours. The result elaborates reduced glucose level as a result of a combination of adiponectin and Metformin.

All data are expressed as mean±SEM.

Table 1: Blood glucose level

	Fasting level	1	2	3	4	5
Control	2.8	8.25	5.51	4.78	4.13	4.17
Metformin	3	3.48	2.78	3.56	3.63	3.68
Metfromin-Adiponectin	2.9	3.98	2.62	2.9	2	2.2

$$df= 6-1=5$$

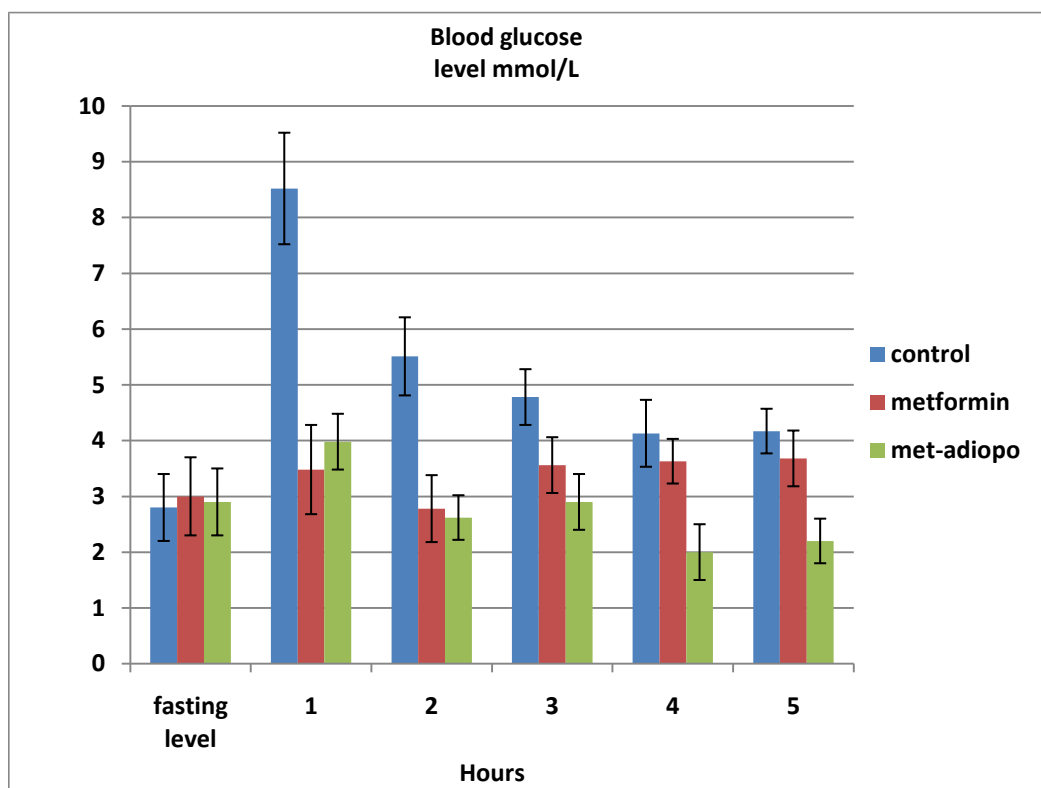


Figure2. The blood glucose were just under 3 mmol/L at fasting level which calculated by glucometer before feeding the experimental mice. The graph indicates a gradual reduction of blood glucose level for Metformin-adiponection group at 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> hours compared with control and metformin groups which were calculated by *t*- test.

### 4.3- Measurement of LDL and HDL in blood

When LDL levels were compared among control, metformin and metformin-adiponectin groups at the end of the fourth hour of the experiment using available commercial kits (table 2), there was no significant difference between three groups ( $p$  value  $> 0.05$ ) (Figure 3a)

However, significant correlation was observed from HDL level between control group and metformin-adiponectin group ( $p$  value  $<0.05$  (Figure. 3b), but no significant difference of HDL level among metformin group and metformin-adiponectin group (table 3) ( $p$  value  $> 0.05$ ) (Figure 3b).

Table 2: HDL level after treatment

HDL level	Control	Metformin	Metformin-Adiponectin
mmol/L	0.91	0.921	0.922

Table3: LDL level after treatment

LDL level	Control	Metformin	Metformin-Adiponectin
mmol/L	0.47	0.53	0.45



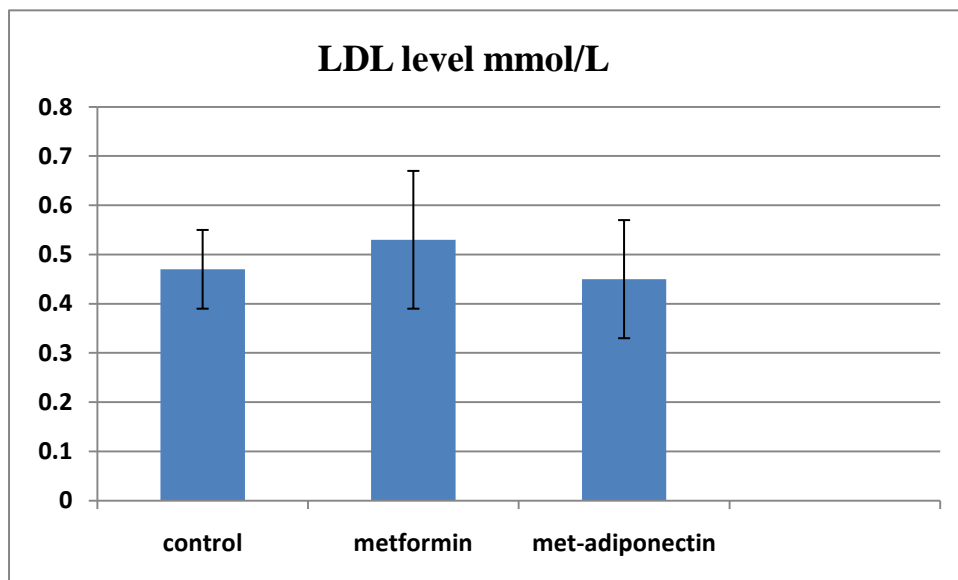


Figure 3a. No significant differences ( $p$  value  $> 0.05$ ) were observed among experimental groups.

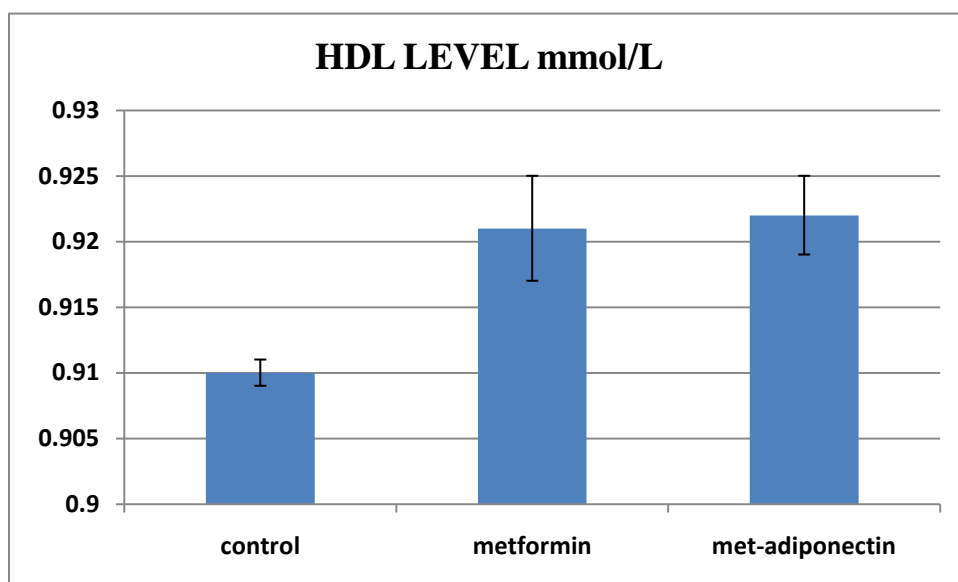


Figure 3b. Significant difference between control and both metformin and metformin-adiponectin groups ( $p$  value  $< 0.05$ )

#### 4.4-Measurement of total Cholesterol & Triglyceride level in blood.

The result of total cholesterol level showed no significant difference between three groups at end of the fourth hour of the experiment ( $p$  value  $> 0.05$ ) (Figure 4a) which means the combination of metformin and adiponectin do not have considerable affect on cholesterol level (table 4 and 5).

Result also showed that although there is no significant difference in the combination of adiponectin and metformin on triglyceride level, compared with metformin alone ( $p$  value  $> 0.05$ ) (Figure 4b), but there is a significant difference between the level of triglyceride in control and both metformin and metformin-adiponectin group ( $p$  value  $< 0.01$ )

Table 4: Triglyceride level

	Control	Metformin	Metformin-Adiponectin
Triglyceride mmol/L	0.8	0.42	0.46

( $p$  value  $> 0.05$ ) No significant difference in combination of metformin and adiponectin in compare with solely metformin.

Table 5: Total Cholesterol level

	Control	Metformin	Metformin-Adiponectin
Cholesterol mmol/L	2	2.2	1.9

( $p$  value  $> 0.05$ ) No significant difference among three groups

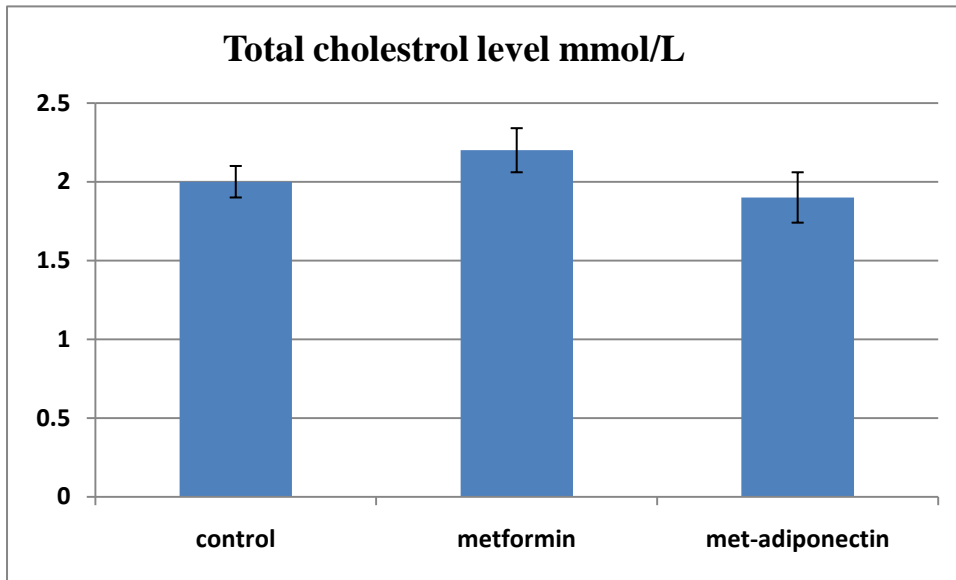


Figure 4a. . TCL show no significant differences ( $p$  value  $> 0.05$ ) between three groups of control, metformin, met-adiponectin.

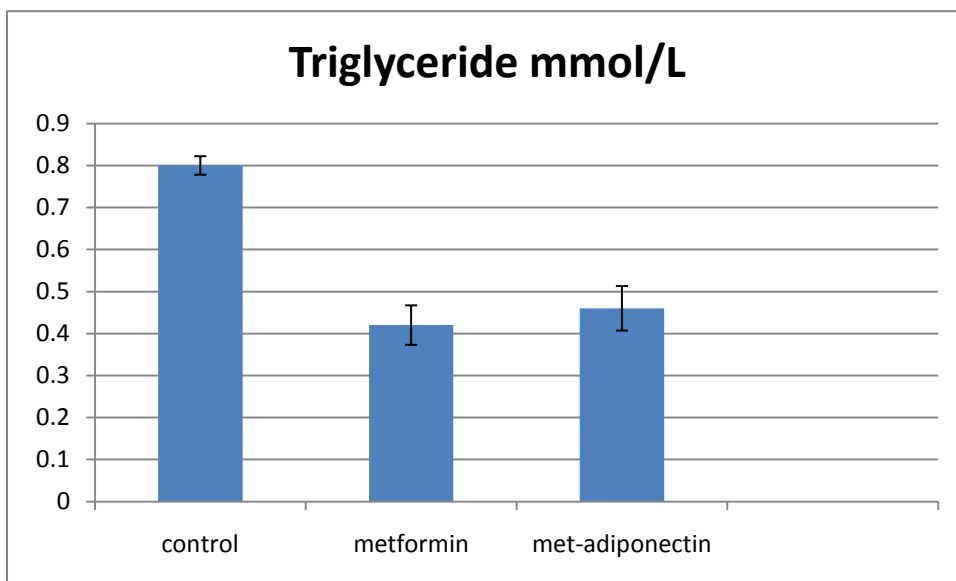


Figure 4b. Triglyceride level between three groups of control, metformin, met-adiponectin show significant difference ( $p$  value  $< 0.01$ )

#### 4.5. Acute toxicity study

An acute toxicity study was carried out in which the mice treated with the adiponectin at a dose of 2mg/kg and 5mg/kg of adiponectin were kept under observation for 14 days. All the animals remained alive and did not manifest any significant visible signs of toxicity at these doses. There were no abnormal signs, behavioural changes, body weight changes, or macroscopic findings at any time of observation. There was no mortality in the above-mentioned doses at the end of 14 days of observation. Histology of liver and kidney, haematology and serum biochemistry revealed no significant differences between groups. From these results it is concluded that the adiponectin is quite safe even at these higher doses and has no acute toxicity and the oral lethal dose (LD<sub>50</sub>) for the male and female mice was greater than 5mg/kg body weight.

##### 4.5.2 Behavioural observation and mortality

The administration of adiponectin did not cause mortality among all groups (Table 6). In addition, no behavioural signs of toxicity were observed throughout the period of the study period among all groups.

**Table 6:** The observation data for toxicology study of adiponectin

Dose	Occurrence of mortality				
	30 min	2 h	4 h	24 h	48 h
5 mg/kg	0/6	0/6	0/6	0/6	0/6
2 mg/kg	0/6	0/6	0/6	0/6	0/6
Vehicle (distilled water)	0/6	0/6	0/6	0/6	0/6

### 4.5.3 Haematology

Total white blood cells for all rats were counted. Table 7 showed that there are no significant differences among all groups.

**Table 7:** Total white blood cell (WBC count ( $10^9/L$ ))

Group	Vehicle (distilled water)	2 mg/kg adiponectin	5 mg/kg adiponectin
Male	$8.45 \pm 1.76$	$7.75 \pm 1.95$	$8.05 \pm 1.62$
Female	$8.28 \pm 2.3$	$7.71 \pm 2.21$	$7.48 \pm 1.34$

All values expressed as  $\pm$  S.E.M.

### 4.5.4 Serum biochemistry

The parameters of liver functions that have been tested were aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin, globulin, total bilirubin(TB), conjugated bilirubin (CB), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT). Their levels were analyzed as indication of liver functions compared to their vehicle group. Furthermore, the level of urea, creatinine, anion gap and serum electrolytes ( $CO_2$ , Potassium, Sodium and Chloride) of all groups were determined as markers of kidneys functions.

Tables 8 and 9 showed that in rats groups were given with SMESE, no significant changes were found in all tested parameters levels as compared to their respective vehicle group.

**Table 8: Acute toxicity test for renal**

Animal Group	Renal Function Test						
	Sodium (mmol/L)	Pottasium (mmol/L)	Chloride (mmol/L)	CO <sub>2</sub> (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (μmol/L)
Normal control	137.75 ± 0.46	5.13 ± 0.19	104.13 ± 0.17	22.13 ± 0.76	18.15 ± 0.76	5.4 ± 0.46	50.73 ± 1.67
L. D (2.5ml/kg)	137.97 ± 0.42	5.26 ± 0.18	102.97 ± 1.25	21.62 ± 0.76	19.17 ± 1.58	4.98 ± 0.47	48.67 ± 0.81
H. D (5mlkg)	138.15 ± 0.42	4.63 ± 0.19	102.86 ± 0.78	23.11 ± 0.97	17.87 ± 0.65	5.5 ± 0.48	48.35 ± 1.83

All values expressed as mean and standards error mean. There is no significant difference between groups.

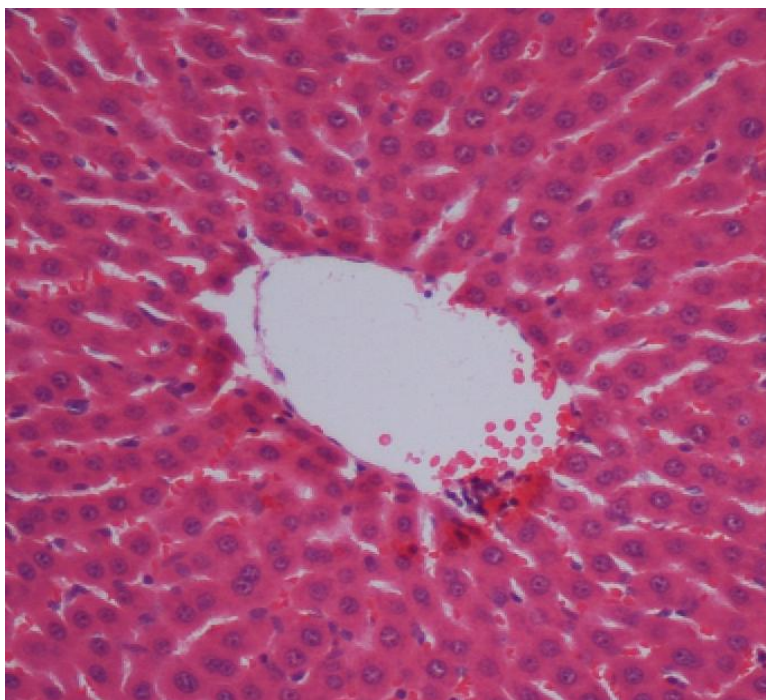
**Table 9: Acute toxicity test for liver**

Animal Group	Liver Function Test								
	TP (g/L)	Albumin (g/L)	Globulin (g/L)	TB ( $\mu\text{mol/L}$ )	CB ( $\mu\text{mol/L}$ )	AP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Normal Control	71.15 $\pm$ 1.61	11.13 $\pm$ 0.61	59.33 $\pm$ 1.66	1.88 $\pm$ 0.16	0.97 $\pm$ 0.13	134.63 + 7.87	53.13 $\pm$ 3.15	151.5 $\pm$ 8.55	4.87 $\pm$ 0.96
L.D (2.5ml/kg)	71.17 $\pm$ 0.5	11.76 $\pm$ 0.33	59.5 $\pm$ 0.33	2.11 $\pm$ 0.17	1.00 $\pm$ 0.00	133.87 + 8.91	50.42 $\pm$ 1.38	154.64 $\pm$ 3.35	5.00 $\pm$ 1.25
H D (5ml/kg)	71.70 $\pm$ 1.03	11.8 $\pm$ 0.7	60.02 $\pm$ 0.85	1.67 $\pm$ 0.26	1.00 $\pm$ 0.00	135.33 + 6.81	52.61 $\pm$ 3.25	155.0 $\pm$ 101.33	5.13 $\pm$ 1.08

All values expressed as mean and standards error mean. There is no significant difference between groups.

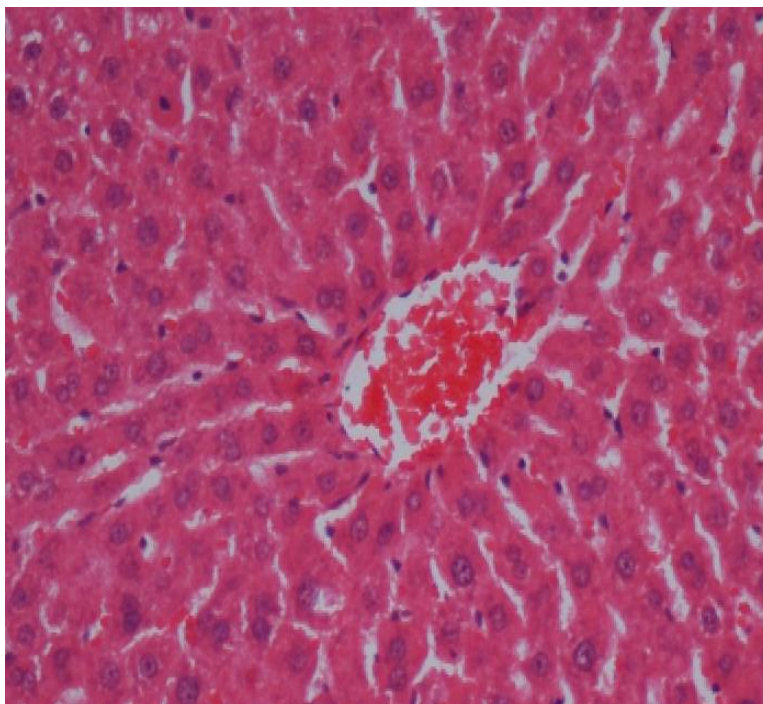
#### 4.5.5 Gross necropsy and histology

There was no significant decrease or increase in levels of all parameters of males as well as females groups in all dosage which are shown in Figure 5.1. (a) to 5.1 (d)

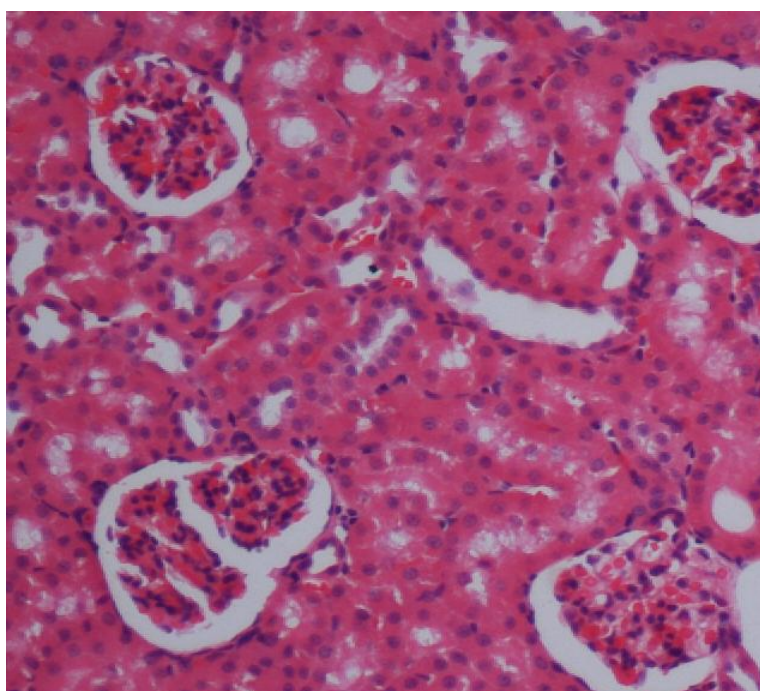


**Figure 5.1a:** Histological section of liver in a rat pre-treated with vehicle (distilled water) showed normal structural appearance. (H&E stain 20x).

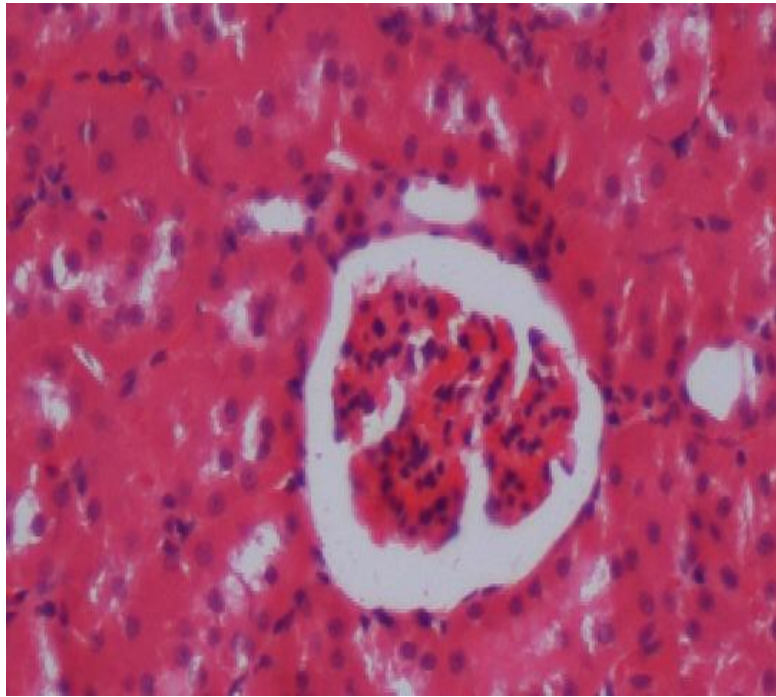




**Figure 5.1b:** Histological section of liver in a rat pre-treated with 5 mg/kg adiponectin showed normal structural appearance. (H&E stain 20x).



**Figure 5.1c:** Histological section of kidney in a rat pre-treated with vehicle (distilled water) showed normal structural appearance. (H&E stain 20x).



**Figure 5.1d:** Histological section of kidney in a rat pre-treated with 5 mg/kg adiponectin showed normal structural appearance. (H&E stain 20x)

## **4.6 Anti-ulcer activity**

### **4.6.1 Gross evaluation of gastric lesions**

The anti-ulcer activity of adiponectin in the ethanol-induced gastric lesion model is reported in Table 10. Results showed that rats pre-treated with adiponectin before being given absolute ethanol had significantly reduced areas of gastric ulcer formation compared to rats pre-treated with only distilled water (ulcer control group) (Figures 5.2.a, b and c) which means to say that the adiponectin significantly suppressed the formation of ulcers. Moreover it was interesting to note the flattening of gastric mucosal folds in rats pre-treated with adiponectin. It was also observed that protection of gastric mucosa was more prominent in rats pre-treated with 5ml/kg adiponectin (Table 10). Besides, ethanol-induced mucosal damage was significant and dose dependently reduced in the size and severity by pre-treatment of the animals with adiponectin. The significant inhibition of gastric ulcer in pre-treatment with adiponectin was compared with omeprazole which is a standard drug used for curing gastric ulcer.

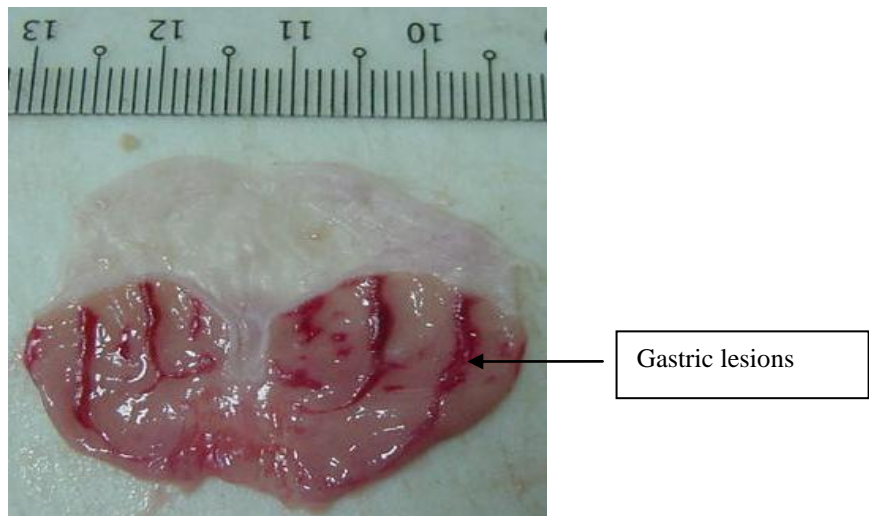
Table 10: Observed ulcer area and inhibition percentage in rats

Animal Group	Pre-treatment	Ulcer area (mm) <sup>2</sup> (Mean ± S.E.M)	Inhibition (%)
1	Distilled water  (Ulcer control)	825.33 ± 11.86 <sup>a</sup>	-
2	Omeprazole  (20mg/kg)	183.00 ± 8.47 <sup>b</sup>	57.79%
3	Adiponectin  (2.5mg/kg)	201.17 ± 9.00 <sup>c</sup>	75.63%
4	Adiponectin  (5mg/kg)	97.33 ± 8.02 <sup>d</sup>	88.23%

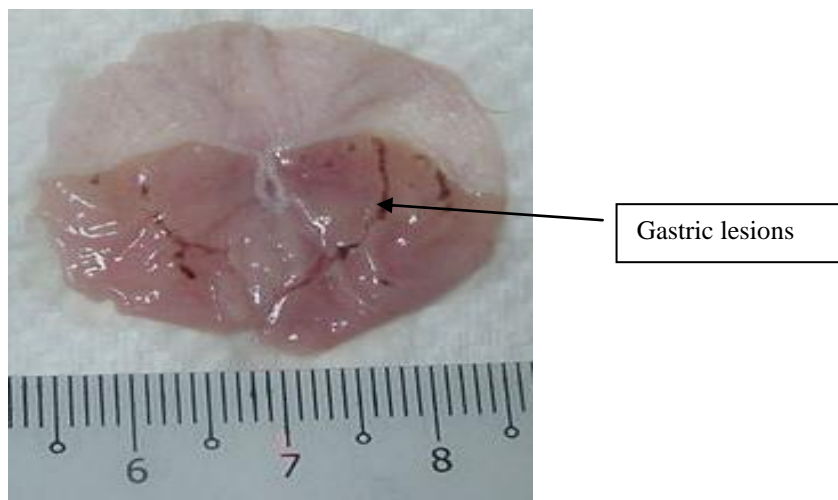
All values are expressed as mean ± standard error mean. Means with different superscripts are significantly different. The mean difference is significant at the 0.05 level.

#### 4.6.2 Histological evaluation of gastric lesions

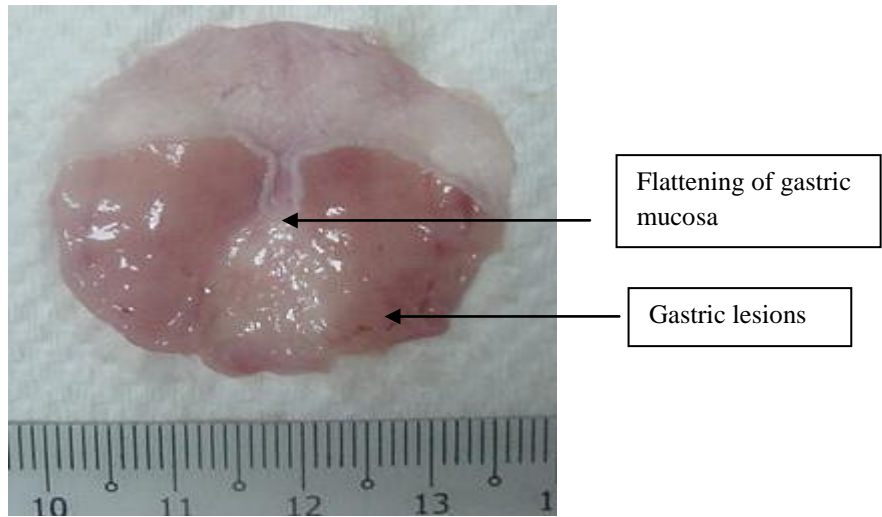
Histological observation of ethanol induced gastric lesions in the ulcer control group pre-treated with only distilled water showed comparatively extensive damage to the gastric mucosa, edema and leukocyte infiltration of the submucosal layer (Figure 5.2.d). Rats that received pre-treatment with adiponectin had comparatively better protection of gastric mucosa as seen by the reduction in ulcer area, reduced or absence of sub mucosal edema and leukocyte infiltration. (Figure 5.2.e and f). The adiponectin has been shown to exert the cytoprotective effects in a dose-dependent manner.



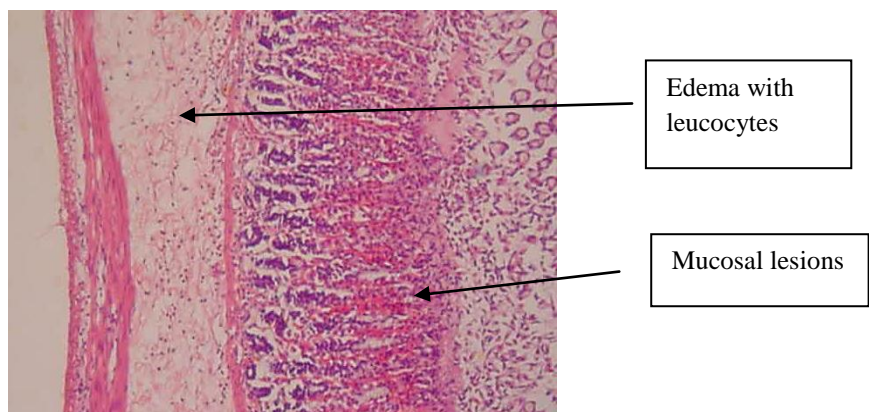
**Fig. 5.2a:** Gross appearance of the gastric mucosa in a rat pre-treated with distilled water (negative control). Severe injuries are seen in the gastric mucosa.



**Fig. 5.2.b:** Gross appearance of the gastric mucosa in a rat pre-treated with omeprazole (20mg/kg). Injuries to the gastric mucosa are milder compared to the injuries seen in the negative control rat.

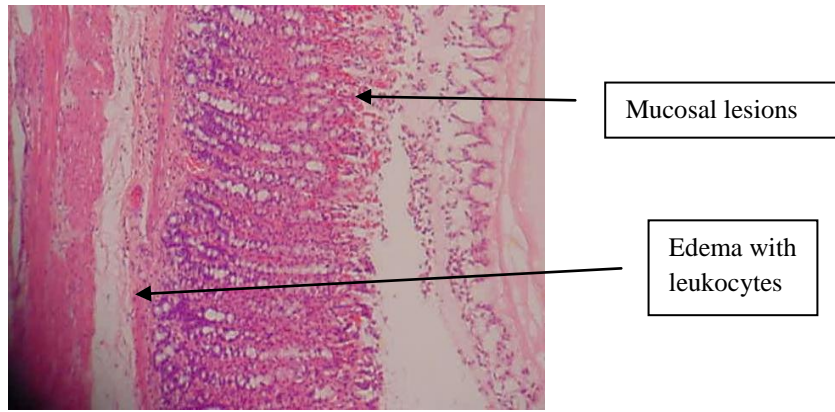


**Fig. 5.2.c:** Gross appearance of the gastric mucosa in a rat pre-treated with 5ml/kg of adiponectin. No injuries to the gastric mucosa are seen, and flattening of gastric mucosa is shown

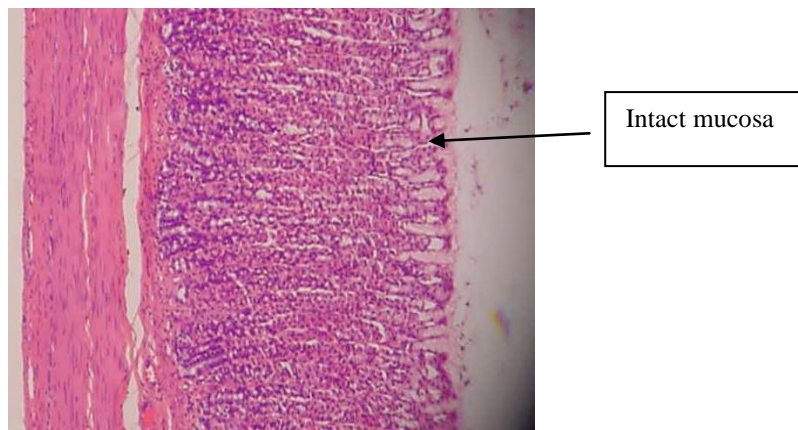


**Fig. 5.2.d:** Histological section of gastric mucosa in a rat pre-treated with distilled water only. There is severe disruption to the surface epithelium, and edema of the submucosa layer with leucocyte infiltration (H&E stain 10x).





**Fig. 5.2.e:** Histological section of gastric mucosa in a rat pre-treated with omeprazole (20mg/kg). There is mild disruption to the surface epithelium with mild edema and leucocyte infiltration of the submucosal layer (H&E stain 10x).



**Fig. 5.2.f:** Histological section of gastric mucosa in a rat pre-treated with 5ml/kg of adiponectin. There is no disruption to the surface epithelium with no edema and no leucocyte infiltration of the submucosal layer (H&E stain 10x).

## **Chapter 5**

# **Discussion**



One of the adipocyte-specific plasma proteins which are secreted by adipose tissue like other adipocytokines, is adiponectin. Adiponectin modulates a variety of metabolic processes, including glucose regulation and fatty acid catabolism (DeGuo *et al.*, 2007). Adiponectin displays a multiplicity of functions, such as antiatherogenic and antidiabetic properties (Matsuzawa, 2005), and also acts as an endogenous regulator of endothelial cells in response to inflammatory stimuli and regulates the metabolism of lipids and proteins (Ekmekci and Ekmekci, 2006). Although adiponectin is secreted from adipose tissue, it is well known that low levels of this protein are present in obese people, which means the levels of this hormone are inversely correlated with body fat percentage in adults (Levri *et al.*, 2005, Kadowaki and Yamauchi, 2005, Casado *et al.*, 2002). Adiponectin is secreted into the bloodstream and it accounts for 0.01% of total plasma protein. Plasma concentrations of adiponectin reveal a sexual dimorphism with males having lower levels than females (Takahashi *et al.*, 2000, Matsuzawa, 2005). A number of oligomeric forms of adiponectin have been described in blood (Wang *et al.*, 2002). Initially three adiponectin molecules bind together to form a homotrimer. Like the plasma concentration, the relative levels of the higher-order structures are sexually dimorphic, where females have increased proportions of the high-molecular weight forms (Diez and Iglesias, 2003).

Production of adiponectin thus is very important and trustworthy for basic research and clinical applications. According to preparation for the operation and production systems compared to recent studies, one of the increasingly popular cellular hosts for expressing the adiponectin is the methylotrophic yeast, *P. pastoris* (Macauley-Patrick *et al.*, 2005). The *P. pastoris* expression systems have been successfully

developed for the production of a variety of heterologous recombinant proteins (Kim *et al.*, 1999, Moller, 2001).

High triglyceride levels and decreased HDL cholesterol levels, but relatively small differences in LDL are the most common patterns of dyslipidemia in type 2 diabetics (Haffner *et al.*, 1992). The mean triglyceride level in type 2 diabetes is <200mg/dl and 85-95% of patients have triglyceride levels below 400mg/dl. In diabetics, optimal LDL cholesterol levels are <100mg/dl, optimal HDL cholesterol levels are >45mg/dl and optimal triglyceride levels are <200mg/dl. The study of (Huypens *et al.*, 2005) indicates that metformin is one of the anti-diabetic drugs which has opposing effects on adiponectin protein expression and releases in differentiated adipocytes.

Since metformin is potent insulin-sensitizing agent that acts primarily on hepatic glucose production and has additional effects on peripheral insulin sensitivity, results from the present study indicate that the effects of a combination of metformin and adiponectin on blood glucose are more effective than the effects of adiponectin itself. The study of adiponectin by Jaleel *et al.* (2006) showed that the values of fasting and random blood glucose, serum triglycerides and LDL cholesterol were significantly increased in diabetic patients as compared with normal control subjects, except for serum adiponectin and HDL cholesterol concentrations which were significantly decreased in diabetic patients (Jaleel *et al.*, 2006).

On the other hand, regarding to (Diez and Iglesias, 2003) the pharmacological effect of adiponectin on dropping insulin resistance is correlated to a decline in triglyceride content and in plasma fatty acid levels in liver and muscle.

Although metformin increases lipolysis and reduces triglyceride stores in adipocytes (Huypens *et al.*, 2005), and triglyceride accumulation is significantly reduced through supporting the oxidation of lipid treatment of diabetic animals with adiponectin, our study shows that there is no significant difference in the combination of adiponectin and metformin on triglyceride, compared with metformin alone (Vionnet *et al.*, 2000). This may be because of suppression of adiponectin production through metformin action in different adipocytes (Huypens *et al.*, 2005).

Since cholesterol levels are closely associated with LDL levels, when total cholesterol levels increased, LDL would do so. Schmitt *et al.* suggested that LDL uptake by fibroblasts may be impaired in type 2 diabetes. This leads to an increase in LDL: HDL ratio in type 2 diabetics. In our study, the LDL ratio did not differ significantly between the control and metformin - adiponectin groups ( $p$  value  $>0.05$ ). It was not found any significant difference in the total cholesterol levels in the metformin- adiponectin group compared to other groups of mice (Schmitt *et al.*, 1982)

Adiponectin has also been proven by recent studies to be a remarkable protective factor against atherosclerosis development due to its anti-inflammatory effects. Now by this theory it is believed that the use of recombinant adiponectin is helpful to avoid cardiovascular disease (Diez and Iglesias, 2003).

It is known that gastric lesions produced by ethanol administration appeared as multiple-hemorrhagic red bands of different size along the glandular stomach. Absolute ethanol is commonly used for inducing ulcer in experimental rats and lead to intense gastric mucosal damage. In the present study, was observed flattening of the mucosal folds which suggests that gastro-protective effect of adiponectin might be due to a decrease in gastric motility. It is reported that the changes in the gastric

motility may play a role in the development and prevention of experimental gastric lesions (Garrick *et al.*, 1986; Takeuchi *et al.*, 1988). Relaxation of circular muscles may protect the gastric mucosa through flattening of the folds. This will increase the mucosal area exposed to necrotizing agents and reduce the volume of the gastric irritants on rugal crest (Takeuchi and Nobuhara, 1985). Ethanol produces a marked contraction of the circular muscles of rat fundic strip. Such a contraction can lead to mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds leading to necrosis and ulceration (Mersereau and Hinchey, 1982).

The result of the present study also revealed protection of gastric mucosa and inhibition of leucocytes infiltration of gastric wall in rats pre-treated with adiponectin. This study evaluated the activity of adiponectin on gastric ulcer compared to omeprazole for the first time. In the present study, the pre-treated rats with adiponectin have significantly reduced areas of gastric ulcer formation before being given absolute alcohol compared to rats pre-treated with only distilled water. This proves that the adiponectin significantly suppressed the creation of ulcers. Moreover, the present study indicated that the protection of gastric mucosa is more prevalent in rats pre-treated with adiponectin. Besides, ethanol-induced mucosal damage is significant and does dependently reduce in size and severity by pre-treatment of the animals with adiponectin. The significant inhibition of gastric ulcer in pre-treatment with adiponectin was compared with omeprazole which is a standard drug used for curing gastric ulcer. In histological observation, the rats that received pre-treatment with adiponectin had comparatively better protection of the gastric mucosa. Subsequently, adiponectin has been shown to exert cytoprotective effects in a dose-dependent manner.

## **Chapter 6**

# **Conclusion**

Adiponectin is one of the most important adipocytokines of adipose tissue. It is plentifully present in human plasma and is vastly expressed and actively secreted by adipocytes. Adiponectin displays a range of functions, such as having antiatherogenic, anti-inflammatory and antidiabetic properties (Matsuzawa, 2005).

Metformin is a potent insulin-sensitizing agent that acts mainly on hepatic glucose production. Metformin has an excellent safety profile and is effective as monotherapy or in combination with sulfonylureas, insulin, and thiazolidinediones (Kirpichnikov *et al.*, 2002).

Our study supports the hypothesis that a combination of adiponectin with metformin might have better effects on blood glucose levels and HDL (Schulze *et al.*, 2004).

Observations from this study suggest that a combination therapy with metformin plus adiponectin is probably attributable to the natural cause of type 2 diabetes rather than to the effect of therapy itself, and requires further clarification (Kirpichnikov *et al.*, 2002, Patane *et al.*, 2000).

Moreover, our data suggests that adiponectin protein plays a significant role on inflammatory diseases such as gastric ulcer, and significantly suppresses the formation of ulcers. A limitation of our study was a comparatively low sample number which results in correlation coefficients which are comparatively weak.

Therefore, More research needs to be done and further investigations involving larger sample numbers are required to confirm the validity of these results.

## **Appendix**

### **BMGY (Buffered Glycerol-complex Medium) & BMMY (Buffered Methanol-complex Medium)**

1% yeast extract

2% peptone

100mM potassium phosphate, pH 6.0

1.34 % YNB

1% glycerol for BMGY or methanol for BMMY

0.5g of yeast extract was dissolved with 1g peptone in 35ml distilled water and mixed well in a Beacker. Solution cooled to room temperature, the following were added and mix well:

5ml potassium phosphate buffer, pH 6.0

5ml 100X YNB

100µl 500X biotin

Beacker was autoclaved for 15min at 121°C and 5ml 10X glycerol for BMGY, 5ml 10X methanol for BMMY was added. Media were stored at +4°C. The shelf life of this solution is approximately two months.

### **Nickel-based purification columns protocol:**

8ml of sodium phosphate buffer was added into purification column and dropping should have finished. Then tubes were centrifuged containing acetone-precipitated sample. 3ml of acetone-precipitated sample were added into column. 10ml of binding buffer (washing step) were added continuously.

Sodium phosphate	10ml
Nacl	14.61gr
Imidazole	5ml
dH <sub>2</sub> O fill up to	500ml

4ml of elution buffer were added and drops were collected.

**For recharging the column:**

8ml of 50m $\mu$  of EDTA

8ml of s dH<sub>2</sub>O

8ml of Nickel

**Contents of SDS-PAGE solution:**

The prerequisites to confirm the size of the adiponectin protein by SDS-PAGE

required the preparation of the following:

1. 30% acrylamide stock
2. 1.5 M Tris-HCl
3. 10% (w/v) SDS
4. 10% (w/v) ammonium persulphate
5. 10% (w/v) TEMED
6. Water-saturated butanol
7. 1 X SDS electrophoresis buffer
8. Gel storage solution
9. Stacking solution

**Acrylamide stock 30%**

	Final concentration	Amount
Acrylamide (Mw 71.08)	30%	300g
Bis N,N' methylenebisacrylamide (Mw 154.17)		8g
Deionised water		up to 1000ml

*(stored at 4°C)*

**1.5 M Tris HCl**

	Final concentration	Amount
Tris (Mw 121.14)	1.5 M	545g
HCl to ph 8.8		about 50ml
Deionised water		up to 3000ml

*(stored at 4°C)*



**10% (w/v) SDS**

	Final concentration	Amount
Sodium Dodecyl sulfate (Mw 288.38)	10%	10g
Deionised water		up to 100ml

*(stored at room temperature)*

**12.5% homogenous gel solution**

Final % T	12.5%
30% acrylamide stock	3330µl
1.5 M Tris-HCl	200µl
dH <sub>2</sub> O	2520ml
10% SDS	50µl
10% TEMED	10µl
10% APS	100µl

**10%(w/v) Ammonium persulphate**

	Final concentration	Amount
Ammonium persulphate (Mw71.08)	10%	0.5g
Deionised water		5.0ml

*(Prepared fresh)*

**10% (w/v) TEMED**

	Amount
TEMED (Mw116.2)	0.5ml
Deionised water	4.5ml

*(Prepared fresh)*

### Water-saturated butanol

	Amount
n-butanol	50ml
Deionised water	10ml

*(Stored at room temperature)*

### Gel storage solution

	Final concentration	Amount
Tris-Cl (1.5M, Ph 8.8)	0.375 M	50ml
10% (w/v) SDS	0.1%	2ml
Deionised water		up to 200ml

*(Stored at 4°C)*

### 1 X SDS electrophoresis buffer

	Final concentration	Amount
Tris (Mw 121.14)	25mM	32.25g
Glycine (Mw 75.07)	192Mm	144g
SDS (Mw 288.38)	0.1% (w/v)	10g
Distilled water		up to 10L

*(Stored at 4°)*

### Infiltrating solution

	Final concentration	Amount
Ethanol	100%	15ml
Sodium acetate		3.4g
Glutaraldehyde		125µl
Sodium thiosulphate		0.1g

**Fixative solution**

	Final concentration	Amount
Ethanol	100%	25ml
Acetic acid		5ml
dH <sub>2</sub> O		up to 50ml

**Silver solutio**

	Final concentration	Amount
Silver nitrate	0.1%	0.05g
Formaldehyde		10μl
dH <sub>2</sub> O		up to 50ml

**Staining solution**

	Final concentration	Amount
Sodium carbonates	0.1%	125g
Formaldehyde		5μl
dH <sub>2</sub> O		up to 50ml

**Contents of Western blot solution:****Blocking solution**

	<u>Amount</u>
1 X TBS	50ml
Skim milk	2.5g

*Keep cold. Can be reused.*

**Probing solution**

	<u>Amount</u>
1 X TBS	6ml

Blocking solution 4ml

**10X Tris-buffered saline (TBS)**

	<u>Amount</u>
Tris base	12.11g
NaCl	87.66g

Tris base was dissolved in 500ml distilled water, and pH was adjusted to 7.4 with HCl. Then NaCl was dissolved and final volume was adjusted to 1000ml with sdH<sub>2</sub>O. The 10X stock solution was diluted to 1X working solution.

**Tris-buffered saline-Tween 20 (TBST)**

	<u>Amount</u>
1X TBS (see above)	500ml
Tween 20	2.5ml

**10X Western transfer buffer**

	<u>Amount</u>
Tris base	22.4g
Glycine	144.1g
sdH <sub>2</sub> O	up to 1000m