Study of GLUT-4 and AMPK gene expression and phosphorylation of AMPK protein in Oyster Mushroom-induced type 2 diabetic model rats

Content

Declaration

Iherebydeclarethat the thesis entitled 'Study of GLUT-4 and AMPK gene expression and phosphorylation of AMPK protein in Oyster Mushroom induced Type 2 diabetic model rats' is myown work and effort under my honorable supervisor**Prof. Dr. Naiyyum Choudhury**, Department of Mathematics and Natural Science, BRAC Universityandsupervisor **Dr. Md Omar Faruque**, Associate professor, Department of Physiology and Molecular Biology, Bangladesh University of Health Sciences. It has not been submitted anywhere for anyaward. Whereothersources ofinformation havebeen used, theyhavebeen acknowledged.

Student name: Md.Asrafuzzaman

ID: 15176003

Signature ……

Supervisor

Prof. Dr. Naiyyum Choudhury
Professor
Department of Mathematics and Natural Science
BRAC University

Signature ……

Dr. Md Omar Faruque
Associate Professor
Department of Physiology and Molecular Biology
Bangladesh University of Health Sciences

Signature ……

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Md. Asrafuzzaman Signature:……

ID: 15176003

Department of Mathematics and Natural Science

BRAC University

Abstract

Type-2 diabetes mellitus is a complex and heterogeneous disorder which is depends upon insulin resistance and later insulin insufficiency and recognize by impaired insulin sensitivity as well as hyperglycemia, thus it represent one of the world leading causes of mortality and morbidity. So drug discovery and development have led to better ways to manage diabetes and its complications. GLUT-4 and AMPK gene has become the focus of the great deal of attention as a novel therapeutic target for the treatment of metabolic syndrome. Oyster Mushroom is traditionally used as remedy of diabetes and hypertension. The present study aims to observe the expression of GLUT-4 and AMPK gene and phosphorylation of AMPK proteins in insulin signaling pathway in STZ induced diabetic model rats through Oyster mushroom administration.STZ induced Type-2 diabetic model rats (Long-Evans) were used in this experiment.Rats were divided in two three groups:Diabetic control, Gliclazide treated rat and Mushroom treated rat.Mushroom was provided as feed supplement with 5% Oyster Mushroom powder. Glucose was measured using glucose oxidase method, lipid profile by enzymatic-colorimetric method, GLUT-4 & AMPK gene expression by rt-PCR methods and specific protein was identified using western blot and immunoprecipitation techniques. RNA extracted by TRIzol reagent and protein extracted by RIPA lysis buffer using different tissues. Fasting serum glucose level was 8.52±2.49 (mmol/L) in mushroom treated groups.On day 42 fasting serum glucose was 6.88±1.17 (mmol/L) and at day 56 it was 5.62 ± 2.02 (mmol/L) respectively.So serum glucose level was found to be decreased gradually but the decrease was non-statistically significant.The serum glucose level of water control diabetic rat on 42 day of also decreased non significantly. However, this low level of glucose did not persist for a longer period andon day 56 it was 4.66 ±1.78 mmol/L compared to 3.84 ±0.611mmol/L on day 42. In Gliclazide treated group fasting glucose concentration were 10.01±3.44, 10.48 ±2.66 and 6.70 ±2.82 on 0 day, 42 day and 56 day respectively. Mushroom treated rats increased total cholesterol level 63% at 42th day but at end of the experiment it became 32%. The standard drug Gliclazide treated group showed a decreased level of both triglyceride 37% and total cholesterol level 17% at the end of the study period.Water Diabetic control rats showed a 11% decrease in triglyceride level & a 42% increase in total cholesterol level on 56^{th} day. The higher expression of genes (GLUT-4, AMPK,) for Mushroom treated rat tissues (Muscle, Adipose, Liver) was observed than non-diabetic rats, control diabetic rats but quit less than gliclazide treated rat.P-AMPK for muscle and

adipose tissue of diabetic control rats seems to be decreased compare to non-diabetic rats. But in case of mushroom treated diabetic rats, p-AMPK have increased compare to control rats. Besides, in case of adipose tissue, p-AMPK seems to decrease compare to non-diabetic rats. Mushroom treated rats has increased its p-AMPK protein better than the gliclazide treated rats. That means Oyster Mushroom can increases GLUT-4 and AMPK gene activity into the cell and thus contribute to reduce hyperglycemic state of type 2 diabetic model rats.

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Chapter One

Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is one of the most common non-communicable diseases (NCDs) globally. It is the fourth or fifth leading cause of death in most high-income countries and there is substantial evidence that it is epidemic in many economically developing and newly industrialized countries. Diabetes is undoubtedly one of the most challenging health problems in the 21st century. The number of studies describing the possible causes and distribution of diabetes over the last 20 years has been extraordinary.These studies continue to confirm that it is the low- and middle-income countries (LMICs) that face the greatest burden of diabetes However, many governments and public health planners still remain largely unaware of the current magnitude or more importantly, the future potential for increases in diabetes and its serious complications in their own countries. More than 80% of diabetes deaths occur in low- and middle-income countries.The World Health Organization (WHO) projects that diabetes deaths will increase by two thirds between 2008 and 2030 [1]

Diabetes mellitus is a metabolic disorder of multiple acetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism.It makes defects of insulin secretion and insulin action or both [2]. Lack of Insulin leads to various metabolic complications in the human body with increases blood glucose, cholesterol, creatinine and transaminases enzyme level and decreases protein content [3].The effect of chronic hyperglycemia causes long term damage, dysfunction and failure of different organs especially the eye, kidneys, nerves, heart and blood vessels [2].The tissue or vascular damage due to this disease progresses ensues leading to severe diabetic complications.Such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration.Thus, diabetes mellitus covers a wide range of heterogeneous diseases [4]. Many pathogenic process are involved in the improvement of diabetes.This range from auto-immune destruction of the beta-cell of the pancreases and resulting in insulin deficiency and in resistance to insulin action [2]. So diabetes mellitus is an important endocrine health problem affecting major population in the world.The World Health Organization (WHO) observed worldwide 170 million diabetic patients in 2002 and predicted the number to be 366 million or more in 2030 [3].

In Bangladesh, which had a population of 149.8 million in 2011, a recent meta-analysis showed that the prevalence of diabetes among adults had increased substantially, from 4% in 1995 to

2000 and 5% in 2001 to 2005 to 9% in 2006 to 2010. According to the International Diabetes Federation, the prevalence will be 13% by 2030. However, no nationally representative, epidemiological study of the prevalence of diabetes mellitus and its risk factors has been carried out in the country [5].Bangladesh is more at risk to develop diabetes, hyperinsulinemia and coronary heart disease compared with other South Asian migrants settled in the UK. Data from previous studies showed a marginal risk for type 2 diabetes with higher BMI for both sexes in Bangladesh. An Indian study has also shown that higher BMI is independently associated as risk for the occurrence of type 2 diabetes among men and women. South Asians, especially Bangladeshi population represents a particular form of physical structure, usually a deposition of fat in the central abdominal region other than extremities. It is postulated that we are representing a distinctive form of type 2 diabetes among a lean population, related to diabetes described in Indian population [6].

1.2 Historical Perspective of Diabetes Mellitus

The first recognition of what came to be known as DM was documented in the Egyptian ancient papyrus, discovered by Georg Ebers in 1862, dating back to 1550 BC which highlighted the first documented cases of DM over 3500 years ago as stated by Ebbell, in 1937 and Tattersall in 2010.Although this may as well represent the first recognition of diabetes, medical historians believe that the first attempt at describing the symptoms of diabetes was made by Aulus Cornelius Celsus (30 BC-50 AD) of Greece. Celsus had described an ailment which presented with excessive urination in frequency and volume, and painless emaciations. Apollonius Memphites, an Egyptian physician at around 230 BC had used the prefix ‘diabetes’ for the first time to denote an excessive passage of urine and ascribed its aetiology to the kidney. At that time, due to lack of evidence, treatment had involved dehydration and phlebotomy (bloodletting) as a method of treatment . In about 500 BC, two Hindu-Indian physicians (Chakrat & Sushrut) recognised that DM was not a single disorder, and they made observations of the sweetness of urine from observing ants congregating around the urine of patients. Although relatively uncommon, as time went on, the recognition and identification of diabetes became apparent. The concept of nature showed varying and intriguing debates and revisions [7]

1.3 Prevalence of Diabetes mellitus

Globally it was estimated that 382 million people suffer from diabetes with a prevalence of 8.3% in 2013 and by 2035 it is expected to be 592 million [8].

IDF REGION	2013 MILLIONS	2035 MILLIONS	INCREASE %
Africa	19.8	41.4	109%
Middle East and North Africa	34.6	67.9	96%
South-East Asia	72.1	123	71%
South and Central America	24.1	38.5	60%
Western Pacific	138.2	201.8	46%
North America and Caribbean	36.7	50.4	37%
Europe	56.3	68.9	22%
World	**381.8**	**591.9**	**55%**

Table 1.1: IDF Regions and global projections of the number of people with diabetes 2013-2035

The number of people with type 2 diabetes is increasing every country, 80% of people with diabetes live in low and middle income countries [8].

Figure 1.1: Prevalence (%) of diabetes (20-79 years) by income group and age

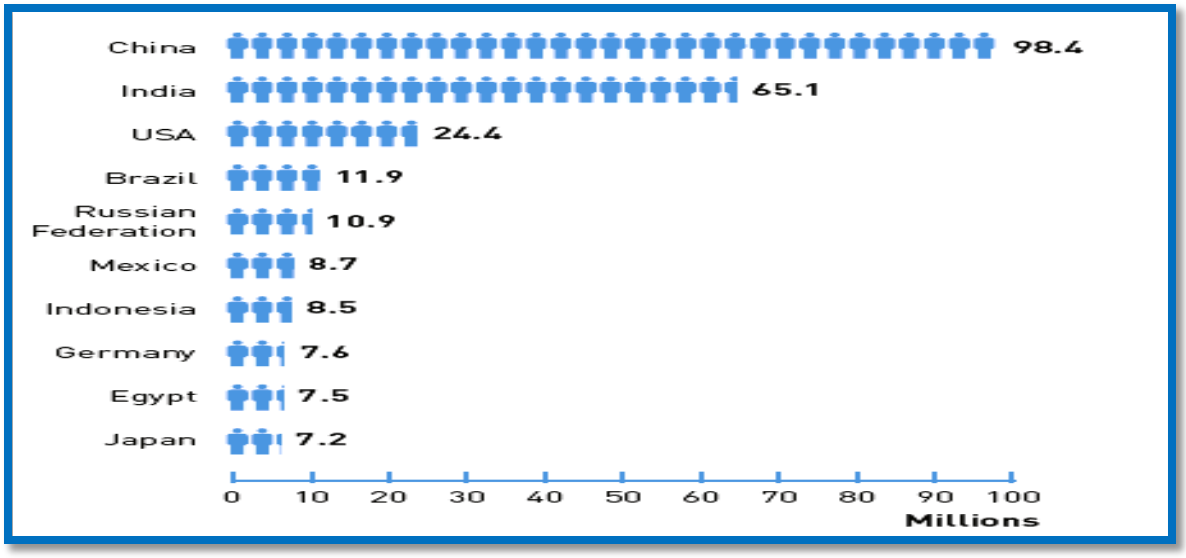


Table 1.2:Top 10 countries of number of people with diabetes (20-79 years), 2013

The greatest number of people with diabetes are between 40-59 years of age [8]. 175 million people with diabetes are undiagnosed in the world.More than 79,000 children developed type 1 diabetes in 2013.More than 21 million live births were affected by diabetes during pregnancy in 2013. Diabetes caused 5.1 million deaths in 2013 Every six seconds a person dies from diabetes[8].Because of its widespread prevalence and potentially debilitating impact, DM has become an international and national priority area of health concern (WHO 2011)

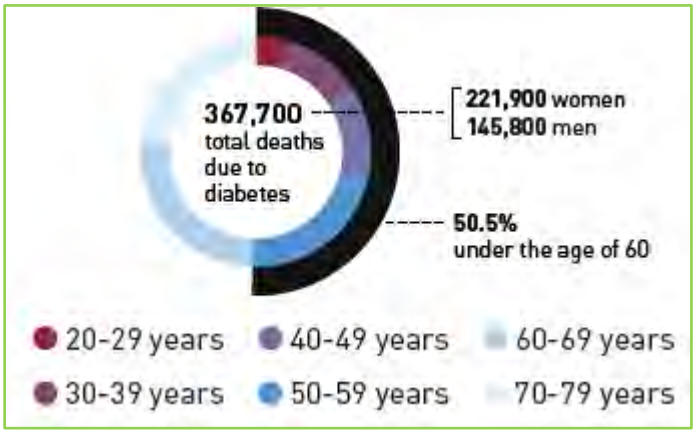


Figure1.2:Deaths due to diabetes by age and sex

In 2011 the International Diabetes Federation (IDF) estimated that 8.4 million of diabetic people in Bangladesh with 8th position in the rank and it is expected to be 16.8 million by the year 2030 with 5th position in rank . It is also estimated that 6.7 million of people living in Bangladesh is suffering from impaired glucose tolerance (IGT). This explosion in diabetes prevalence will place in Bangladesh among the top 5 countries in term of the number of people living with diabetes in 2030.Although the major type of diabetes are type-1 and type-2, but later one is predominant in worldwide [9]. Bangladesh is a United Nations-designated least developed country (LDC) with a disproportionately high diabetes population. Among all people living with diabetes in the 48 LDCs, more than one-third live in Bangladesh.40% of all people with diabetes in least developed countries live in Bangladesh [10].

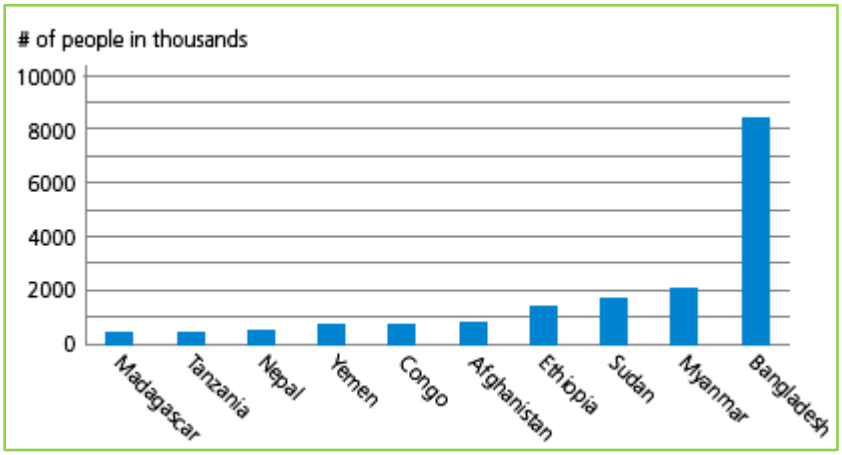


Figure1.3: Distribution of people with diabetes in least developed countries

In type-1 diabetes patient have little or no endogenous insulin and therefore insulin therapy require survival.Type-2 diabetes mellitus is a disease in which insulin is abnormally secreted or does not act properly which lead to elevated blood glucose and prolonged elevated glucose level can lead to multiple organ damage [9]. A recent study investigate the association of diabetes with a broad range of health conditions. People with diabetes had a 25-75% higher risk of dying from cancer, infection, liver disease, lung disease, mental disorder, etc. Overall approximately 40% of excess death in diabetic patients appears to be due to non vascular events [11].

1.4 Classification of diabetes mellitus

Classification of diabetes mellitus is based on its aetiology and clinical presentation. As such, there are four types or classes of diabetes mellitus. Such as type 1 diabetes, type 2 diabetes, gestational diabetes, and other specific types [12].The three main types of diabetes – type 1 diabetes, type 2 diabetes and gestational diabetes –occur when the body cannot produce enough of the hormone insulin or cannot use insulin effectively. Insulin acts as a key that lets the body's cells take in glucose and use it as energy. People with type 1 diabetes, the result of an autoimmune process with very sudden onset, need insulin therapy to survive.Type 2 diabetes, on the other hand, can go unnoticed and undiagnosed for years. In such cases, those affected are unaware of the long-term damage being caused by their disease. Gestational diabetes, which appears during pregnancy, can lead to serious health risks to the mother and her infant and increase the risk for developing type 2 diabetes later in life [8].

1.4.1 Type 1 diabetes mellitus (Insulin dependent diabetes mellitus)

Insulin dependent diabetes mellitus most commonly afflicts juveniles but it can also occur in adults. The disease is characterized by an absolute deficiency of insulin caused by massive beta cell lesions or nacrosis.Los of beta cell function may be due to invasion by viruses, the action of chemical toxins or usually through the action of autoimmune antibodies directed against the beta cell.As a result of the destruction of beta cells, the pancreas fails to respond to ingestion of glucose. Type 1 diabetes require exogenous insulin to avoid hyperglycemia and life threatening ketoacidosis [13]

1.4.2 Type 2 diabetes mellitus (non-insulin dependent diabetes mellitus)

Type 2DM is a chronic, complex disorder which adversely affect both longevity and equality of life due to multiple ,potential, serious complications.It is non ketotic form of diabetes which is not linked to HLA markers on the 6^{th} chromosome and is not associated with islet cell antibodies.The patient are not dependent on exogenous insulin therapy to sustain life-thus it was named as non insulin dependent diabetes mellitus which presently called as type 2 DM [2].

The adult human pacrease consist of many type of cells called islets of Langerhans. On the basis of staining characteristics and appereance, there are four major cell types in the islets of Langerhans [14]

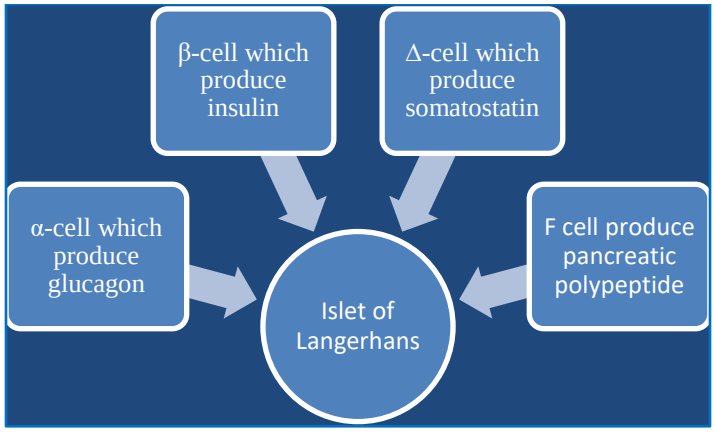


Figure 1.4: Islet of Langerhans

Most important regulator of insulin secretion is a direct response of plasma glucose on the β-cells.GLUT 2 is the glucose transporter of β-cell. The glucose inter the β cell through GLUT 2 and metabolized by glucokinase.Thus ATP is released and this causes ATP sensitive K+ channels closed. This causes potassium efflux is decreased and make cell membrane depolarised. It makes voltage gated calcium channels open and resulting rapid entry of calcium into the β cell.The increased intracellular calcium release insulin by exocytosis forms the granules of the β cell [14].

1.4.2.1 Genetics of type 2 diabetes

Diabetes is a disease that has a strong clustering in families and has a genetic component. It has been widely reported that the occurrence of T2DM is triggered by a genetic susceptibility and familial aggregation in several populations [15].

1.4.2.1.1. Familial aggregation

Family history is a well-known risk factor for the developing of T2DM. It was estimated that risk for diagnosed T2DM increases approximately two to four fold when one or both parents are affected. Almost 25% to 33% of all T2DM patients have family members with diabetes. Having a first degree relative with the disease poses a 40% risk of developing diabetes. T2DM patients

are more likely to have diabetic mothers than diabetic fathers. Family history reflects both inherited genetic susceptibilities and shared environments which include cultural factors. Thus, family history of diabetes may be a useful tool to identify individuals at increased risk of the disease and target behavior modifications that could potentially delay disease onset and improve health outcomes [15]. The diabetic patients enrolled in CODIAB study, 66% had at least one diabetic relative among their parents, aunts, uncles, siblings, or offspring. This frequency can be compared with the diabetes prevalence generally found in the French population. This result supports the familial aggregation of diabetes and suggests that a dominant mode of transmission could be involved [16].

1.4.2.2 Gene discovery of type 2 diabetes

In contrast to monogenic disorders, where results from single mutations lead to predictable phenotypes, the complex genetic architecture of susceptible and protective alleles in polygenic type 2 diabetes is more difficult to discern. Indeed, accumulating data suggest that type 2 diabetes is likely a collection of many closely related diseases with varying but often overlapping primary mechanisms that involve both impaired insulin secretion and insulin resistance. Adding to the challenge, type 2 diabetes is generally diagnosed later in life as a consequence of significant interactions of life-long environmental influences with multiple genetic factors. Because of the limited individual impact of single genetic loci, a full understanding of the complex gene-gene and gene-environment interactions in this disease has proven quite challenging. Prior to the GWAS era, the importance of genetic factors in the etiology of T2DM had been well established through family and twin studies.The primary methods to identify susceptibility loci for diseases or phenotypic traits were linkage analysis and candidate gene association studies [17]

1.4.2.2.1 Linkage study

In the first phase of diabetes gene discovery, investigators used techniques based on linkage analysis to identify potential diabetes-associated genes. This approach, best suited for discovering genes with strong effects within relatively small family-based studies, involves genotyping affected family members for a set of markers to identify regions that are coinherited more commonly in affected family members and therefore potentially point to a genomic region

containing a susceptibility locus [17].The genome-wide linkage approach led to the identification of several loci, the most prominent being the TCF7L2 (Transcription Factor 7 Like 2) gene on chromosome 10q25.3. TCF7L2 has been replicated in almost every population [18].Well-replicated linkage on chromosome 20q resulted in the identification of noncoding variants in the *HNF4*α gene, which have been replicated in some studies.Similarly, linkage with type 2 diabetes or related traits in chromosome 1q (q21 – q23) has been observed across ethnic groups and in multiple populations and encompasses over 400 expressed genes, including many strong candidates. Finally, a thorough exploration of multiple (and modest) linkage signals by deCODE investigators resulted in the identification of the gene encoding the transcription factor 7-like 2 (*TCF7L2*) as the locus that confers the strongest effect on type 2 diabetes risk yet found.This strong association has been replicated in many ethnic groups (with an OR of about 1.4 per risk allele).Multiple risk allele appear to have an additive effect, one allele confers approximately 40% relative risk of diabetes, whereas two copies confer 80% relative risk.Five SNPs and 1 tetranucleotide repeat polymorphism (DG10S478) within *TCF7L2* showed strong association with T2DM in 3 independent cohorts, and the SNPs (rs12255372 and rs7903146) showed strong linkage disequilibrium (LD) with composite at-risk alleles of the microsatellite marker (DG10S478). The association between the SNPs (rs12255372 and rs7903146) and decreased insulin secretion was also reported in American subjects with impaired glucose tolerance.The precise mechanisms by which *TCF7L2* variants increase risk are not well understood, although various lines of evidence suggest that they involve the enteroinsular axis, impaired insulin secretion and possibly reduced β cell proliferation.Although this approach had proven extremely successful in identifying rare genetic variants of strong effects for single-gene disorders such as maturity onset diabetes of the young (MODY), but proved limited in unveiling common genetic variants that underlie polygenic diseases [17].

1.4.2.2.2 Candidate gene of type 2 diabetes

Candidate genes are previously discovered genes that, based on their inferred physiologic role, are hypothesized to contribute to the disease of interest if abnormal. In the case of type 2 diabetes, defects in genes encoding proteins that play a role in pathways involved in insulin control and glucose homeostasis would all be considered reasonable candidates for contributing to the genetic basis of disease. A powerful approach to finding such defects is the identification

of a significant association between diabetes mellitus and a functional polymorphism in a candidate gene. Generally, this is achieved by comparing a random sample of unrelated type 2 diabetes mellitus patients with a matched control group. This approach may show a polymorphic allele that is increased in frequency in the patient group and such a significant association might point towards a disease susceptibility locus. To date, over 250 candidate genes have been studied for their role in type 2 diabetes mellitus [17]. The candidate gene approach led to the identification of two T2D genes now considered widely replicated: PPARG and the β-cell potassium channel (Kir6.2) gene, KCNJ11 [18]. The majority of these studies have failed to uncover any association, possible explanation for this include small sample sizes, differences in T2DM susceptibility across ethnic groups, variation in environmental exposures, gene-environment interaction and in part due to adoption of low statistical thresholds before declaring association. Still, some findings stood the test of time such as role for some of the gene products involved in insulin secretion or insulin action, such as IRS-1, the glucagon receptor, the sulphonylurea receptor (SUR) and the peroxisome proliferator activated receptor-γ(PPARγ) [17]. Because of the current controversy regarding validity of association of many described gene(s), this will focus only on a few of the most promising candidate genes that have been convincingly associated with T2DM:

PPARγ (peroxisome proliferator-activated receptor-γ): This gene has been widely studied because it is important in adipocyte and lipid metabolism. In addition, it is a target for the hypoglycemic drugs known as thiazolidinediones. A proline-to-alanine change in codon 12 (P12A) of the peroxisome proliferator–activated receptor γ (*PPAR*γ) gene was the first genetic variant to be definitively implicated in the common form of type 2 diabetes and is very common in most populations [17]

***ABCC8* (ATP binding cassette, subfamily C, member 8):** This gene encodes the high-affinity sulfonylurea receptor (SUR1) subunit that is coupled to the Kir6.2 subunit (encoded by U*KCNJ11*U, also known as the potassium channel, inwardly rectifying subfamily J, member 11). Both genes are part of the ATP-sensitive potassium channel, which plays a key role in regulating the release of insulin and glucagon in the beta cell. Mutations in either gene can affect the potassium channel's activity and insulin secretion, ultimately leading to the development of T2DM. Interestingly, *ABCC8* and *KCNJ11* are only 4.5 kb apart, and not far from the *INS* gene.

Variant forms of *KCNJ11* (Lys) and *ABCC8* (Ala) genes have been associated with T2DM, as well as other diabetes-related traits [17].

***CAPN10* (calpain 10):** *CAPN10* encodes an ubiquitously expressed intracellular calcium-dependent cysteine protease. A haplotype that was initially linked to T2DM included an intronic A to G mutation at position 43, which appears to be involved in *CAPN10* transcription. Two amino acid polymorphisms (Thr504Ala and Phe200Thr) have also been associated with T2DM risk. It has been suggested that the coding and noncoding polymorphisms do not independently influence T2DM risk, but instead contribute to an earlier age at diagnosis. Physiological studies suggest that variations in calpain 10 activity effects insulin secretion, and therefore, susceptibility to T2DM [17]

***HNF1B*:** Researchon this MODY (maturity onset diabetes of the young) gene has produced a conclusive association of an intronic SNP (rs757210) in hepatocyte nuclear factor 1b (*HNF1B*) (previously known as *TCF2*) with type 2 diabetes [17]

The Glucagon Receptor (GCGR): Glucagon is a key hormone in the regulation of glucose levels. As such, the GCGR gene which encodes its receptor is a candidate diabetes susceptibility gene. A missense mutation in the glucagon receptor gene has been associated with decreased tissue sensitivity to glucagon and type 2 diabetes. One of the major drawbacks of the candidate gene approach is that it will not lead to the identification of entirely new genes or pathways involved in T2DM. In order to identify new genes for T2DM, genome wide scans using polymorphic markers need to be performed [17]

Table 1.3: Genetic Variants Associated with Type 2 Diabetes Mellitus

***Gene Region*, Name (Chromosome Number)**	**Function**
TCF7L2, Transcription factor 7-like 2 (10)	Encodes a high mobility group box- containing transcription factor that plays a key role in *Wnt* signaling pathway.
PPARG, Peroxisome proliferator-activated receptor γ (3)	Transcription factor involved in adipocyte development.
KCNJ11, Potassium inwardly-rectifying channel, subfamily J, member 11 (11)	Potassium channel that is part of the sulfonylurea receptor complex.
WFS1, Wolfram syndrome 1 (4)	Endoplasmic reticulum transmembrane protein expressed in the brain, heart and β-cells.
HNF1B, Hepatocyte nuclear factor-1β (17)	Transcription factor involved in pancreatic development.
SLC30A8, Solute carrier family 30 (zinc transporter), member 8 (8)	Expressed in β-cells – it is a Zinc transporter, this being necessary for insulin storage in secretory granules as well as being part of the secretory mechanism.
HHEX, Hematopoietically expressed homeobox (10)	Encodes a member of the homeobox family of transcription factor involved in pancreatic development.
CDKAL1, CDK5 regulatory subunit associated protein 1-like 1 (6)	The protein product shares homology with CDK5 regulatory subunit–associated-protein-1, a neuronal protein that inhibits activation of CDK-5.
IGF2BP2, IGF-2 mRNA binding protein 2 (3)	Regulates IGF-2 translation by binding to the 5′ UTR of IGF-2 mRNA; pancreatic development.
CDKN2A/B, Cyclin-dependent kinase inhibitor 2A and 2B (9)	Function as cell growth regulators that control cell cycle G1 progression by inhibiting CDK; islet development.
FTO, Fat mass and obesity associated (16)	Affects fat mass thereby indirectly predisposing

	to type 2DM
JAZF1, Juxtaposed with another zinc finger gene 1 (7)	Encodes a nuclear protein with three zinc fingers; functions as a transcriptional repressor.
CDC123-CAMK1D, Cell division cycle 123 homolog (*S. cerevisiae*) and Calcium /calmodulin-dependent protein kinase 1D (10)	CDC123 is a putative regulator of the cell-cycle while CAMK1D is a protein kinase that may be important in response to chemokines.
TSPAN8-LGR5, Tetraspanin 8 and Leucine-rich repeat- containing G protein- coupled receptor 5 (12)	Tetraspanin 8 is a cell surface glycoprotein that complexes with integrins regulating development and growth. Lgr5 is a potential marker of intestinal stem cells and hair follicles in humans. It is a target of *Wnt* signaling.
THADA, Thyroid adenoma associated (2)	Thyroid adenoma; associates with *PPARG*
ADAMTS9, ADAM metallopeptidase with thrombospondin type 1motif, 9 (3)	A member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family, expressed in muscle and pancreas.
NOTCH2, Notch homolog 2 (1)	Transmembrane receptor implicated in pancreatic organogenesis
KCNQ1, Potassium voltage-gated channel, KQT-like subfamily, member 1 (11)	Pore-forming subunit of voltage-gated K-channel (*KvLQT1*); risk allele impairs insulin secretion.
IRS1, Insulin receptor substrate (2)	Plays a key role in transmitting signals from the insulin & IGF-1 to intracellular pathway.
MTNR1B, Melatonin receptor 1B (11)	Encodes one of two high affinity forms of a receptor for melatonin; risk allele associated with insulin secretion.
PROX1, Prospero protein homeobox 1 (1)	Corepressor of hepatocyte nuclear factor 4α which plays an important role in β-cell development
GCKR, Glucokinase regulator (2)	Regulatory protein that inhibits glucokinase.

ADCY5, Adenylate cyclase 5 (3)	Formation of adenylate cyclase.
UBE2E2, Ubiquitin conjugating enzyme E2F2 (3)	
BCL11A, B-cell lymphoma/ leukemia 11A (2)	Encodes C2H2 type zinc-finger protein.
GCK, Glucokinase (7)	Three tissue-specific forms phosphorylate glucose to produce glucose-6-phosphate in the liver and the β-cell.
DGKB/TMEM195, Diacylglycerol kinase beta and Transmembrane protein 195 (7)	DGKB encodes an isotype of DAG kinase which increases DAG and therefore increases insulin secretion. TMEM195 is a membrane phosphoprotein.
*C2CD4A/B*C2, calcium-dependent domain containing 4B (15)	Encodes nuclear localized factor 2 which is expressed in endothelial cells and the endocrine and exocrine pancreas.
KLF14, Kroppel like factor 14, also known as Basic transcription element binding protein (BTED5) (7)	Regulates the transcription of various genes, including TGF βR11.

1.5 Expression of GLUT-4 gene for management of diabetes mellitus

GLUT4 is one of 13 sugar transporter proteins (GLUT1- GLUT12, and HMIT) encoded in the human genome that catalyzes hexose transport across cell membranes through an ATP-independent, facilitative diffusion mechanism. GLUT4 is highly expressed in adipose tissue and skeletal muscle, but these tissues also express a selective cohort of these other transporters. However, GLUT4 displays the unique characteristic of a mostly intracellular disposition in the unstimulated state that is acutely redistributed to the plasma membrane in response to insulin and other stimuli[19]. The increased translocation of GLUT4 by insulin is the major mechanism for glucose uptake, the amount of GLUT4 protein in the intracellular compartment is also important. Indeed, studies from human type 2 diabetes (T2DM) or animal models suggestthat GLUT4 gene expression is controlled at the transcriptionallevel. Tissue-specific knockout of the GLUT4 gene resulted in insulin resistance, while overexpression of GLUT4 resulted in the restoration of whole body glucose disposal and insulin sensitivity. Because skeletal muscle is the major tissue

for glucose disposal, most studies regarding gene expression have been performed in this tissue. Although adipose tissue accounts for only a small portion of glucose disposal, it is reported that transcription of theGLUT4 gene in adipose tissue is decreased both in the insulindeficient model of b-cell destruction and type 2 diabetes model, overweight or type 2 diabetes mellitus patients (T2DM), suggesting the involvement of adipose tissue in insulin sensitivity. Moreover, adipose tissue-specific GLUT4 knockout mice revealed decreased glucose tolerance and insulin sensitivity, while overexpression improved insulin resistance. These results imply that adipose tissue-specific GLUT4 gene expression also plays a pivotal role in glucose homeostasis. Thus, understanding the regulation mechanism of GLUT4 gene expression both in muscle and adipose tissue may provide valuable information in the development of anti-obesity or antidiabetes drugs [20].

1.6 Regulation of protein activation and diabetes mellitus

Protein kinases make up one of the largest gene families in eukaryotes. They are involved in amyriad of cellular processes, and their misregulation often results in disease. There are currently at least forty-six unique protein kinase crystal structures, twenty-four of which are available in an active state [21].

Glucose is the primary fuel for many organisms. The abilityof single cells and whole animals to respond to change inglucose availability is of particular importance for survival. Elevated glucose concentrations stimulate the transcription of the pre-proinsulin (PPI), L-type pyruvate kinase (L-PK), and other genes in islet beta cells. In liver cells, pharmacological activation by 5-amino-4-imidazolecarboxamide riboside (AICAR) of AMPactivated protein kinase (AMPK), the mammalian homologue of the yeast SNF1 kinase complex, inhibits the effects of glucose, suggesting a key signaling role for this kinase. AMPK activity is inhibited by elevated glucose concentrations in MIN6 beta cells. AMPK activity are necessary and sufficient for the regulation of the L-PK gene by the sugar and also play an important role in the regulation of the PPI promoter. AMPK kinase and AMPK are the upstream and downstream components, respectively, of a highly conserved protein kinase cascade.AMPK is now considered as a key metabolic master switch, regulating carbohydrate and fat metabolism in response to change in cellular energy charge, being activated in an ultrasensitivemannerby a fall in ATP/AMP ratio. The enzyme exists as a heterotrimer of α- (catalytic), β- (adapter), and γ- (regulatory) subunits.

Unlike α1 complexes, complexes containing the α2 isoform of the catalytic subunit are found in both the nucleus and the cytoplasm. The regulation of the AMPK α2-subunit may be necessary and sufficient to explain the effects of glucose on L-PK gene expression in beta cells, and also plays an important, but not exclusive, role in the control of insulin promoter activity [22]. Adenosine 58- monophosphate-activated protein kinase (AMPK) now appears to be a metabolic master switch, phosphorylating key target proteins that control flux through metabolic pathways of hepatic ketogenesis, cholesterol synthesis, lipogenesis, and triglyceride synthesis, adipocyte lipolysis, and skeletal muscle fatty acid oxidation. Recent evidence also implicates AMPK as being responsible for mediating the stimulation of glucose uptake induced by muscle contraction. Type 2 diabetes mellitus is likely to be a disease of numerous etiologies. However, defects or disuse (due to a sedentary lifestyle) of the AMPK signaling system would be predicted to result in many of the metabolic perturbations observed in Type 2 diabetes mellitus. Increased recruitment of theAMPK signaling system, either by exercise or pharmaceutical activators, may be effective in correcting insulin resistance in patients with forms of impaired glucose tolerance and Type 2 diabetes resulting from defects in the insulin signaling cascade [23].

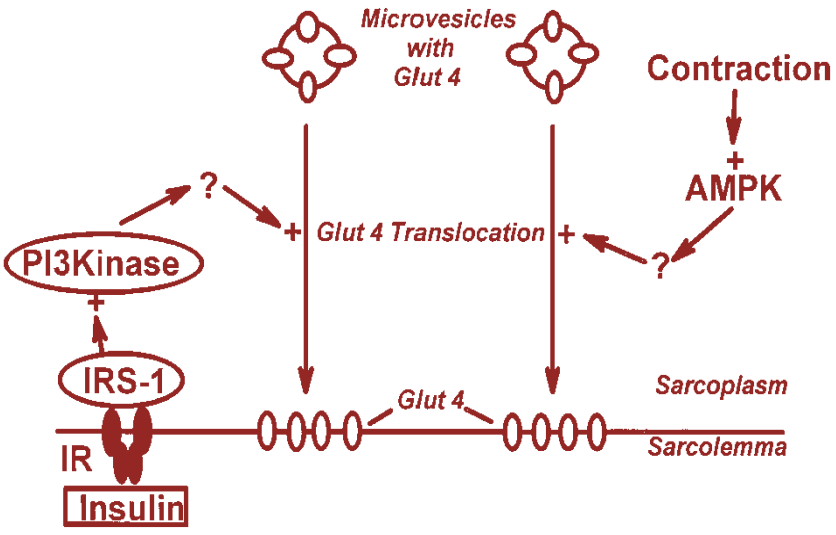


Figure. 1.5. Two mechanisms for stimulation of glucose uptake in skeletal muscle, one mediated by insulin and one triggered by muscle contraction. The hypothesis of mediation of the contraction effect by AMPK is based on the observations that exercise and electrical stimulation increase AMPK activity and glucose uptake.

1.6.1 Subunit domain organization and AMPK regulation

One of the most profound features of AMPK as a metabolic sensor is its sensitivity to the cellular energy status, which results from its unique biochemical properties. AMPK is a heterotrimeric protein consisting of a catalytic α and regulatory β and γ subunits . Each α and β subunit is encoded by 2 genes (α1 and α2 or β1 and β2), whereas the γ subunit is encoded by 3 genes (γ1, γ2, and γ3).

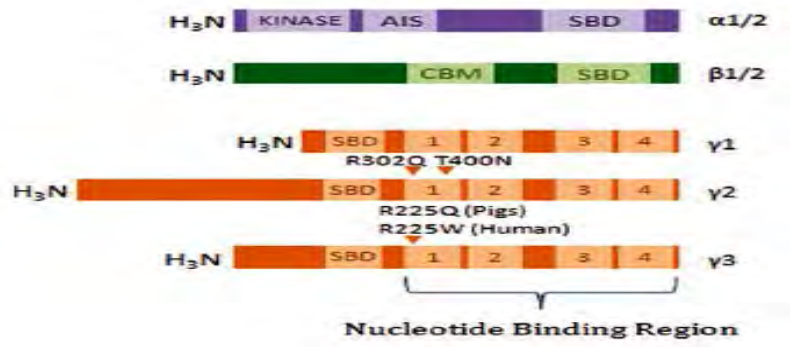


Figure 1.6: α, β, and γ AMPK subunits and isoforms with important domains

The protein is activated in response to an increase in the ratio of AMP to ATP within the cell and therefore acts as an efficient sensor for cellular energy state. Binding of AMP activates AMPK allosterically and induces phosphorylation of a threonine residue (Thr-172) within the activation domain of the α subunit by an upstream kinase, the tumor suppressor LKB1 .Furthermore, binding of AMP inhibits the dephosphorylation of Thr-172 by protein phosphatase, whereas a high concentration of ATP inhibits the activation of AMPK. Activation of AMPK by CaMKK is triggered by a rise in intracellular calcium ions, without detectable changes in the AMP/ATP ratio. CaMKK is highly expressed in the CNS, and lower levels are detected in other tissues such as liver and skeletal muscle, suggesting that the AMPK pathway is regulated by multiple mechanisms that are likely to be tissue specific.

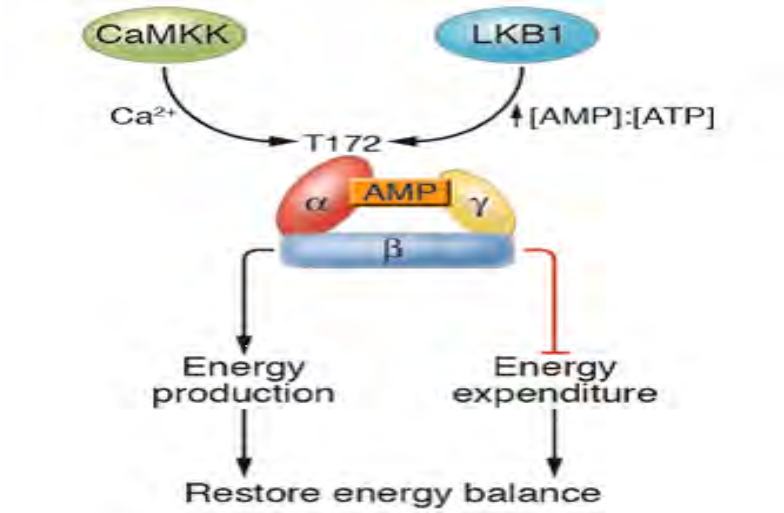


Figure 1.7 :Structure and regulation of AMPK

AMPK activity is activated by a wide array of metabolic stresses, including hypoxia, ischemia, and oxidative and hyperosmotic stresses . Furthermore, exercise and glucose deprivation also activate AMPK, which suggests a role in exercise adaptations and β cell function. In general, activation of AMPK triggers catabolic pathways that produce ATP, while turning off anabolic pathways that consume ATP, to maintain cellular energy stores[24].

1.7 Function of AMPK

AMPK is part of a cell's response to stresses that deplete ATP and threaten its viability.More specifically, they proposed that AMPK acts a''fuel gauge''that when activated stimulates processes that generate ATP and inhibits others that consume ATP, but are not acutely necessary for survival. Early studies revealed that such activation of AMPK occurs in response to a wide variety of factors that deplete cellular energy stores, including glucose deprivation, hypoxia, ischemia and muscle exercise. Among the events simulated by AMPK activation are fatty acid oxidation, glucose transportand glycolysis. Among the processes inhibited at least for finite periods are the synthesis of triglycerides and proteins. As shown in Figure.1.6, rising intracellular AMP displaces ATP bound to the g-subunit of AMPK, altering its structure. It is thought that this structural change may make AMPK less susceptible to dephosphorylation and inactivation by protein phosphatases and more susceptible to activation and phosphorylation on Thr172 in its a-subunit.To date, two such AMPK kinases have been identified. One of them is

LKB1, a tumor suppressor that appears to mediate AMPK activation in response to changes in energy state, and the other calmodulin-dependent protein kinase kinase-b (CAMKK-b), an enzyme that is activated by Ca2" . In contrast to LKB1, CAMMK-b does not appear to require a change in energy state to activate AMPK[25]

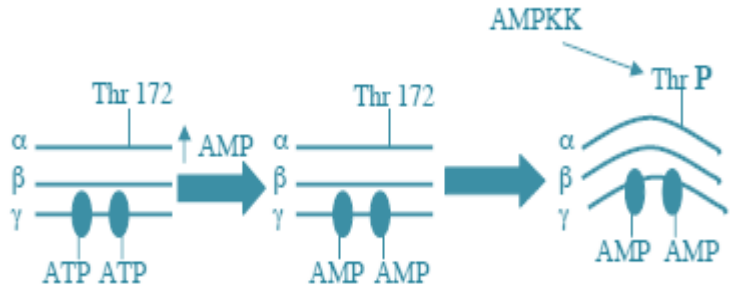


Figure 1.8. Activation of AMP-activated protein kinase (AMPK). ATP binds to specific domains on the gsubunit of AMPK; however, when intracellular adenosine monophosphate (AMP) increases (due to glucose deprivation, hypoxia, etc.) ATP is displaced by AMP owing to its higher binding affinity. The binding of AMP alters the structure of AMPK, thus making it a better substrate for at least one upstream kinase (labeled AMPKK), the tumor suppressor LKB1.

Recently it has been demonstrated that AMPK can also be activated by leptin and a number of other hormones. Furthermore, it has been shown that a lack of these hormones or in some instances their receptors predisposes to obesity, insulin resistance, inflammation and, in genetically predisposed animals and humans, to diabetes and other disorders associated with the metabolic syndrome[25].

1.7.1 Regulation of AMPK

The AMPK system is activated by 58-AMP not only via an allosteric mechanism but also via phosphorylation of a key threonine residue on the catalytic subunit catalyzed by a distinct upstream kinase. Because the latter effect involves a covalent change in the kinase, by use of appropriate conditions it can be preserved during preparation of cell-free extracts, unlike the allosteric effect. Activation is achieved by no less than four mechanisms, such as

- Allosteric activation of AMPK by AMP
- Binding of AMP to AMPK, making it a better substrate forAMPKK
- Binding ofAMPtoAMPK, making it a worse substrate for protein phosphatases, especially protein phosphatase-2C
- And allosteric activation of the upstream kinase, AMPKK, by MP

The combined effect of these mechanisms allows .200-fold activation and results in the system being exquisitely sensitive to small changes in AMP concentration in the cell. All of the activating effects of AMP are antagonized by high concentrations of ATP , so that the system responds to the AMP-to-ATP ratio (AMP/ATP) rather than just to the level of AMP. AMP and ATP vary reciprocally in cells because of the action of adenylate kinase (myokinase), and this may be another device to increase the sensitivity of the system. Recently, it has also been found that physiological concentrations of phosphocreatine allosterically inhibit AMPK. Because it decreases during muscle contraction,phosphocreatine rather than AMP may be the key regulator of the AMPK system during short-term exercise. UnlikeAMP, phosphocreatine does not appear to regulate phosphorylation by the upstream kinase. Because the effects of phosphocreatine on AMPK are entirely allosteric, they would therefore be expected to be lost on preparation of cell-free extracts[23].

1.7.2 Management of type 2 diabetes by targeting AMPK pathway: An emerging concept

AMPK is a global target as it regulates different diversified signals in metabolic pathways. On the basis of the merits associated with this target, an ideal AMPK activator is expected to increase muscle glucose transport and muscle insulin sensitivity; enhance fat oxidation in muscle and liver; inhibit hepatic gluconeogenesis; decrease cholesterol and triglyceride synthesis in liver and should be devoid of problems associated with present antidiabetic drugs (gastrointestinal problem, body weight increase, etc.). Three different kinds of AMPK activators have been reported so far. First, PPARγ activators, rosiglitazone and pioglitazone, which activate AMPK without direct binding but by increasing cellular AMP/ATP ratio. Second, AICAR, an analogue of natural activator AMP, which activates AMPK through direct binding followed by allosteric modification. Lastly, metformin, an AMPK activator which does not affect AMP/ATP ratios or bind to AMPK, but acts through an unknown mechanism. PPARγ agonist rosiglitazone, the

leading anti-diabetic drug, although an activator of AMPK, is associated with PPARγ related side effects, like weight gain and oedema. The anti-diabetic effect of metformin is explained partially through AMPK but it also has several deficiencies. AICAR showed promise in preclinical model as an anti-diabetic drug but failed in phase 1 clinical trial (100 mg/kg). Although it showed good plasma glucose and TG reduction, it produced uric acid and lactic acidosis due to the formation of purine metabolite. Therefore, it is a great challenge to get a safe but efficacious AMPK activator. We also need to remember that there are certain difficulties associated with AMPK, which makes it a difficult pharmacological target [26],

(i) AMPK is a heterotrimeric protein and so far no crystal structure is available

(ii) Each subunit contains two or more isoforms

(iii) The AMP binding site is not well defined.

1.8. Generating cDNA from an RNA population

The field of human genetics was revolutionized whenWatson and Crick described the structure of DNA as a doublehelix in 1953, 74 years after the first DNA isolation by JohannFriedrich Miescher.Several methods have been employed in dentistry tobetter understand and diagnose of different disease. These include culturemethods, microscopy, immunofluorescent assays,enzymelinkedimmunosorbent assays and DNA probes. Morerecently the polymerase chain reaction (PCR) wasintroduced. PCR is a highly sensitive and specific techniqueby which minute quantities of specific DNA (or RNA afterreverse transcription – RT-PCR) can be enzymaticallyamplified [27].The first step in the RT-PCR reaction is to selectively convert only the RNA molecules that correspond to protein-encoding genes into cDNA. The RNA that encodes the protein sequenceis called messenger RNA (mRNA) and it is purified and extracted as a fraction of total cellularRNA from a collection of cells or tissue. Experimentally, the process of reverse-transcription hasmany variations but the essential step is the conversion of mRNA into a cDNA template in areaction catalyzed by an RNA-dependent DNA polymerase enzyme called reverse-transcriptase. A short DNA molecule termed an oligodeoxynucleotide primer is hybridized tocomplementary mRNA that allows the reverse-transcriptase enzyme (RT) to extend the primer andproduce a complementary DNA strand. The sequence of the DNA primer can be designed to bindto a particular target gene or to all mRNA in a sample of purified mRNAs. A

specific syntheticantisense oligonucleotide that hybridizes to the desired mRNA sequence is required for conversionof a specific gene sequence into cDNA. [28]

1.9. Plant materials as a traditional therapy for the control of diabetes

The Bangladeshi traditional medicine is a unique conglomerate of different ethnomedical influences. Due to the geographic location and sociocultural characteristics of the country, it involves traditionally rooted elements influenced by local indigenous .Given its inexpensive, easily accessible and well-established health services, the use of traditional medicine is an integral part of public health services in Bangladesh with its providers being deeply embedded within the local community [29]. Bangladesh is full of medicinal plants, which are used by the people for the treatment of various diseases even at this modern era.Traditionally various plants are being used to treat diabetic patients. In Bangladesh about 5 million people are affected with diabetes.Some plants which are being used to control blood glucose level, such as neem, agrimoney, aloe, bilberry, cinnamon, fenugreek, American ginseng, Asian ginseng, garlic, vincarosea, bitter melon, onion etc. Diabetes is one of the leading causes of death in human and animals [30]. Medicinal mushrooms have been valued as a traditional source of natural bioactive compounds over many centuries and have been targeted as potential hypoglycemic and anti-diabetic agents. Bioactive metabolites including polysaccharides, proteins, dietary fibres, and many other biomolecules isolated from medicinal mushrooms and their cultured mycelia have been shown to be successful in diabetes treatment as biological antihyperglycemic agents [31].

1.9.1.White Oyster Mushroom (*Pleurotus Florida*)

Mushrooms have been widely used as food or food ingredients in many food products for a long time. Some edible mushrooms have been used because of their antitumour, antifungal, and reducing hypercholesterolemia activities. From a nutritional point of view, mushrooms contain high protein and low fat. In addition, high dietary fibre of mushrooms was reported to function as an antitumour and antiviral agent [32].Many of mushrooms pose a range of metabolites of intense interest to pharmaceutical e.g. antitumour, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antiplatelet-aggregating,antihyperglycaemic, antimicrobial and antiviral activities (antitumour, immunomodulation agents, and hypocholesterol-aemic agents) and food (e.g. flavor compound)

industries [33]. Moreover, mushrooms are recognized as a good source of amino acids which play an important role in their flavours. *Pleurotus* mushrooms, commonly known as oyster mushrooms, grow wildly in tropical and subtropical areas, and are easily artificially cultivated. They are healthy foods, low in calories and in fat, rich in protein, chitin, vitamins and minerals [32]. A high nutritional values of oyster mushrooms has been reported with protein (25-50%), fat (2-5%), sugars (17-47%), mycocellulose (7-38%) and minerals (potassium, phosphorus, calcium, sodium) of about 8-12% [2]. Edible mushrooms are also rich in vitamins such as niacin, riboflavin, vitamin D, C, B1, B5 and B6 [33]. They also contain high amounts of -amino butyric acid (GABA) and ornithine. GABA is a nonessential amino acid that functions as a neurotransmitter whereas ornithine is a precursor in the synthesis of arginine.There are about 40 species of *Pleurotus* mushrooms and they rank second among the important cultivated mushrooms in the world[32]. Different types of synthetic oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, but have side effects, including hematological coma and disturbances of liver and kidney. Hence, the search for effective anti-diabetic agents of natural origin that could be used on a long term basis without any side effects is a priority[32].White Oyster Mushrooms appear to be effective for both the control of blood glucose and the modification of the course of diabetic complications without side-effect [35].

1.9.2. Rational study of Oyster Mushroom

The use of mushrooms in traditional ancient therapiesdates back at least to the Neolithic age. For millennia,mushrooms have been valued as edible and medical provisionsfor humankind[36].Medicinal mushrooms have been identified as remarkable therapeutic agents in traditional folk medicines andimportant as popular as culinary products all over the world.Species of medicinal mushrooms have a long history of use for disease treatmentin folk medicines, especially in countries such as China,India, Japan and Korea [37]. In Eastern Countries like China and Japan the knowledge on the use of edible and medicinal mushrooms had been passed on from one generation to the other in documentedform. For example, over 2,500 years ago, many medicinal mushrooms had been recorded and depicted in the earliest Chinesematerial medica book, *Shennong Bencao Jing*, and other succeeding Chinese medical book.Information on the indigenous use of mushrooms had been passed orally from one generation to another [38].The variation of climatic conditions within the continentis directly

reflected on the prevalence of mushrooms indifferent geographical regions. Accordingly, medicinal applicationsmay differ not only between different countries, butalso between different ethnic groups inhabiting the samecountry. Mushrooms belonging to species of *Termitomyces,Pleurotus, Lentinus, Lenzites, Trametes, Ganoderma, Pycnoporus,Coriolopsis*, and *Calvatia* have been reported to beused in folk medicine in Nigeria. On the otherhand, mushrooms of the species *Termitomyces, Agaricus,Boletus, Pleurotus, Cantharellus, Macrolepiota, Ganoderma,*and *Geastrum* have been reported in Tanzania.Historically, the first reports record the application of mushroom as a hallucinogenic agent by the people of the Yorubatribe in Nigeria. Additionally, theYoruba traditional doctors applied a medicinal preparationof *Termitomyces microcarpus* for the treatment of gonorrhea [39].There is a common saying that "medicines and foods have a common origin".Dietary mushrooms provide a wide variety of medicinalproperties and they are effective against certain lifethreateningdiseases. Major medicinal properties attributedto mushrooms include anticancer, antibiotic, antiviralactivities, immunity and blood lipid lowering effects.Oyster mushrooms are very effective in reducing the total plasma cholesterol and triglyceride level and thus reduce the chance of atherosclerosisand other cardiovascular and artery related disorders.These medicinal properties might be due to thepresence of some important substance in dietary mushrooms[40].Mushrooms are incredibly popular foods and have been valued as remedies for various diseases in numerous countries throughout the world. Medicinal mushrooms thereby provide a rich reservoir for the development of new therapeutic agents. The biologically active metabolites and components derived from medicinal mushrooms have demonstrated beneficial effects on diabetes through the regulation of several pathophysiological pathways related to the onset of diabetes. Some of the antihyperglycemic mechanisms of medicinal mushrooms have been investigated including β-cell improvement and insulin releasing activity, antioxidant defenses, carbohydrate metabolism pathways, α-glucosidase and aldose reductase inhibitory activities .Mushrooms have immense potential and may be developed as effective and safe anti-diabetic therapy though detailed studies are still needed for the isolation and production of novel anti-diabetic compounds from mushrooms [41].

1.10. Hypothesis

Oyster Mushroom is associated with the activation of AMP-activated protein kinase (AMPK) for the treatment of type 2 diabetes.

1.11 Objective

General objective

The aim of the study was to understand the genetic and proteomic basis of ant diabetic effect of Oyster Mushroom on the activation of AMP-activated protein kinase pathway.

Specific Objectives

1. To observed the effect of Oyster Mushroom on glycemic status in type 2 diabetic model rats.
2. To observed the effect of Oyster Mushroom on GLUT-4 and AMPK gene expression.
3. To know the effect of Oyster Mushroom on the phosphorylation of AMPK protein in type-2 diabetic model rats.

Chapter Two

Methods & Materials

2.1 Place of the study

The study was conducted in Bangladesh University of Health Science at department of Physiology & Molecular Biology, Dhaka, Bangladesh. The study was done during the period of 9 months (May 2015 to February 2016).

2.2.Preparation of rat feed supplemented with 5% Oyster Mushroom (*Pleurotus ostreatus)* powder.

All the ingredients of standard rat pallet i.e. Flour,Wheat bran, Maize bran, Rice bran, Fish meal, Beshon, Powder milk, Salt, Oil, Vitamin, Molasses, Oil cakewere purchased from the market for poultry feed. Oyster Mushroom (*Pleurotus ostreatus*) was collected also purchased from local market. Then all the ingredients were mixed up in a bowl with the following proportions Flour 35%, Wheat bran 15%, Maize bran 8%, Rice bran 4%, Fish meal 10%, Beshon 3%, Powder milk 4%, Salt 0.5%, Oil 1%, Vitamin 1%, Molasses 0.5%, Oil cake 8% and Oyster Mushroom 5%. Water was added to make a dough and then the dough was placed in a oven tray with a thin layer and dried in the oven at 150^0C for 30 min. This feed supplemented with 5% Oyster Mushroom was prepared everyday throughout the experimental period.

Figure 2.1: Standard pellet food

2.3. Animal

Adult long Evans rats weighting 170-220g was used in this study. The animals were breed at Bangladesh University of Health Science animal house, Dhaka ,Bangladesh, maintained at constant room temperature of 22°C with humidity of 40-70% and natural 12 hours day-night cycle.The experiment was conducted according to the ethical guidelines approved by Bangladesh Association for Laboratory animal science.

2.3.1 Preparation of type 2 diabetes model rats

Type 2 diabetes model rat was created by a single intraperitoneal injection of streptozotocin (STZ) in citrate buffer (P^H 4.5) at a dose of 90mg/kg of body weight into the rat pups (48 hr. old, average weight 7 gm). Following 3 month of STZ injection rat were examined for their blood glucose level by oral glucose tolerance test (OGTT). Diabetic model rats having blood glucose level > 7.00 mmol/l, at fasting condition were selected for studying the effect of white Oyster Mushroom powder.

Figure 2.2:Injection of Streptozotocin to create Type 2 model.

2.3.2Formulation of gliclazide and Mushroom powder doses

As a positive control for this study, thestandard drug gliclazide was prepared at a dose of 20 mg per 5 ml of solvent (Water +few drops of 1N Sodium Hydroxide)/kg body weight of T2DM rat models and administered orally.For evaluation of the anti diabetic activity, the Oyster mushroom powder was supplemented with food to the rats for 8 weeks (56 days) at a ratio of 5g mushroom powder per 100g feed.

2.3.3 Dose and route of administration

For all the pharmacological studies, the drug gliclazide administrated orally at a dose of 20 mg/5 ml/kg body weight to Type 2 diabetic model rats. For the control groups, normal food was given.

2.4. Experimental design

A total of 20 (2 Normal rats and 18 type 2 diabetic model rats) rats were used in this 60 days chronic experimental period.

Group -1 (n=6)	Type 2 diabetic model rats control group, water administered as vehicle [7.5g food/100g body weight/day]
Group-2 (n=6)	Type 2 model rats positive control group, standard drug gliclazide was administered [20 mg per 5 ml of solvent (Water +few drops of 1N Sodium Hydroxide)/kg body weight]
Group-3 (n=6)	White Oyster Mushroom treated group

Table 2.1. Experimental groups

2.5. Collection of blood sample for biochemical analysis

Blood samples were collected from rats kept under fasting conditions by amputation of the tail tip under diethyl ether anesthesia at 0^{th} day for four times with 30 minute intervals. Just before the amputation, the tail was immersed into warm water (about 40°C) for approximately 20 - 30 seconds for vasodilatation. After cutting the tail tip, about 0.2 ml blood was taken cautiously in micro centrifuge tube to avoid haemolysis. On the 42^{th} and 56^{th} day, after the animals were decapitated, their blood was collected from heart.

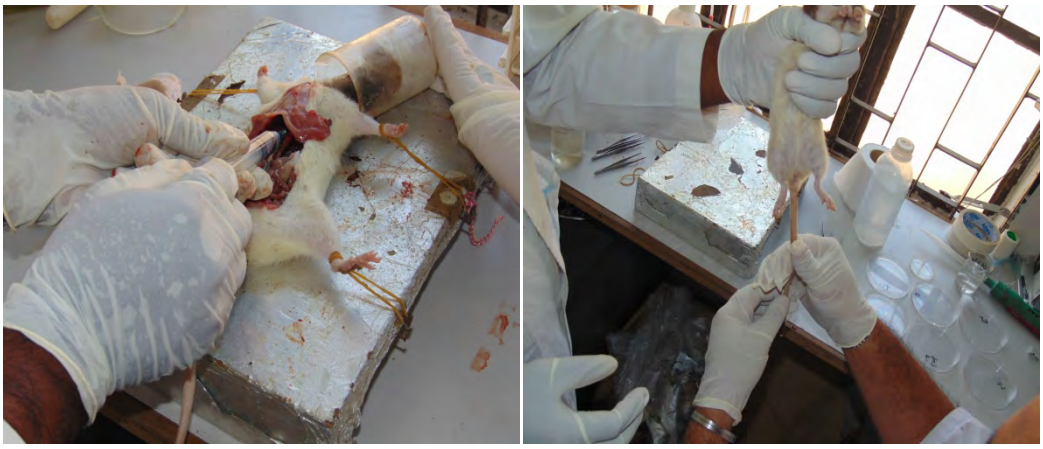


Figure 2.3: Blood collected from heart and tail tip after amputation.

Collected blood samples were centrifuged at 2500 rpm for 15 minutes and finally the serums were separated into another micro centrifuge tubes for biochemical analysis and 100µl of serum were kept frozen at -20°C until analysis of fasting serum insulin.

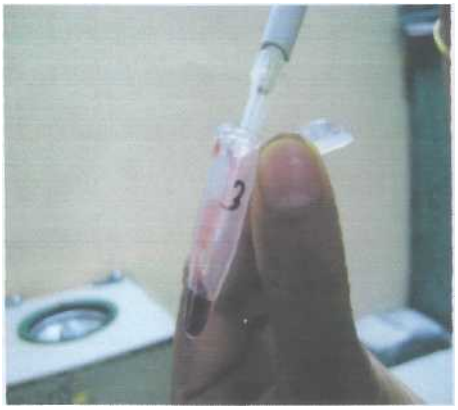


Figure 2.4: Separation of serum after centrifugation from the blood

2.6. Recording of body weight

All the rats were provided with constant environmental condition and were provided with enough food and water throughout the experiment.The body weights of each rat were measured every seven days interval and accordingly they were fed with their respective treatment.

2.7.Biochemical analysis

The following parameters of type 2 diabetic model rats were measured for the anti- diabetic effects of *Mushroom.*

- Serum glucose was measured by Glucose Oxidase (GOD-PAP) method using micro-plate reader (Bio-Tec, ELISA).
- Serum total cholesterol by enzymatic colorimetric (Cholesterol Oxidase/ Peroxidase, CHOD-PAP) method (Randox Laboratories Ltd., UK), using autoanalyzer, AutoLab.
- Serum triglyceride (TG) by enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) using autoanalyzer, AutoLab.

2.7.1 Serum glucose estimation by glucose oxidase (GOD-PAP) method (Boehringer-Mannheim Gmbh)

Principle

The Aldehyde group of p-D-glucose is oxidized by glucose oxidase to produce gluconicacid and hydrogen peroxide. Hydrogen peroxide is further broken down to water and oxygen in the presence of peroxidase and in presence of an oxygen acceptor (i.e. phenol) to produce a colored compound. The reaction of GOD-POD reagent with glucose produces 4-aminophenazone, a red colored compound [42].

Glucose oxidase

P-D-glucose + O2 + H2O $\longrightarrow$ Gluconic acid + H2O2

Peroxidase

2H2O2 + 4-aminophenazone + Phenol $\longrightarrow$ 4-(p-benzoquinone- monoimino) phenazone + H2O

Reagents composition

Buffer: Phosphate buffer (0.1 mol/1, ph 7.0),Phenol (llmol/1)
GOD-POD Reagent: 4-aminophenazone (0.77 mmol/1),Glucose oxida(>1.5 kU/1)Peroxidase (>1.5 kU/1)
Standard Glucose (5.55 mmol/1).
Procedure

While pipetting into the wells, the first two wells were kept blank and the 7 standard glucose solutions (5µl) of each concentration were pipetted in the next 7 wells of the micro-plate with duplicates. The serum samples (5µl) were pipette in the remaining micro-wells of the plate, each of them were pipeted twice. GOD-PAP reagent (250µl) was next added in all the wells. The plate was then incubated in Labsystems iEMS Shaker incubator for proper dilution for 15minutes at 37°C and using the Ultra micro- plate ELISA Reader (Bio-Tek ELx 808 USA), the absorbance of the samples were read.

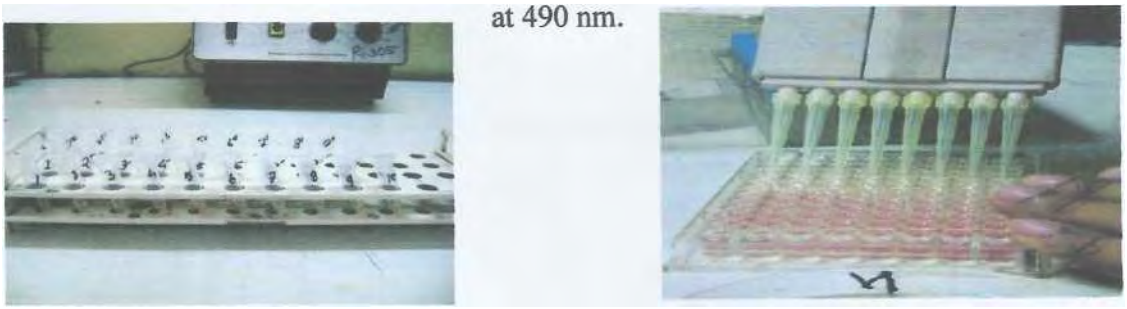


Figure 2.5: Serum samples of rat and GOD-PAP reagent Being added to all the samples for determination of glucose.

Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs. concentration of the standard solutions against a reagent blank. Based on the calibration curve, the unknown concentrations of glucose in theserum sample were

measured maintaining the same mixing and incubation conditions asfor the standard solutions. The standard curve was drawn parallel on every experiment day.

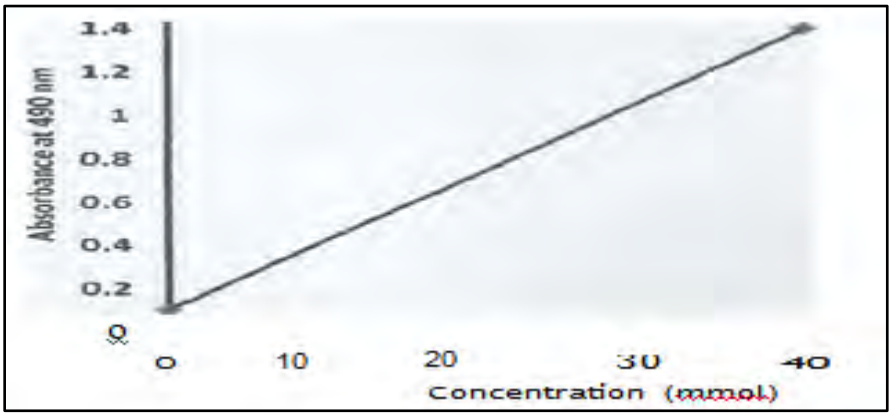


Figure 2.6: Standard curves for glucose

2.7.2. Estimation of serum total cholesterol by enzymatic colorimetric (cholesterol oxidase / peroxidase) method

Principle

The Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxiee and 4-aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids.}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimmine} + 4H_2O$$

All reagents purchased from RANDOX, UK for the determination of the levels of cholesterol in rat serum samples.

Content (Reagent)	Initial Concentration of solution
4-Aminoantipyrine	0.25mmol/l
Phenol	6mmol/l
Peroxidase	>0.5U/ml
Cholesterol esterase	>0.15U/ml
Cholesterol oxidase	>0.1U/ml
Pipes Buffer	80mmol/l; pH 6.8
Standard	5.17 mmol/l

Table 2.2: Reagent for determining cholesterol

Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Dimension LR, Siemens, UK)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Automatic Analyzer. 5µl sample and 200µlreagent were mixed and incubated at 37°C for 5 minutes within the Auto Analyzer. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula and expressed in mg/dl.

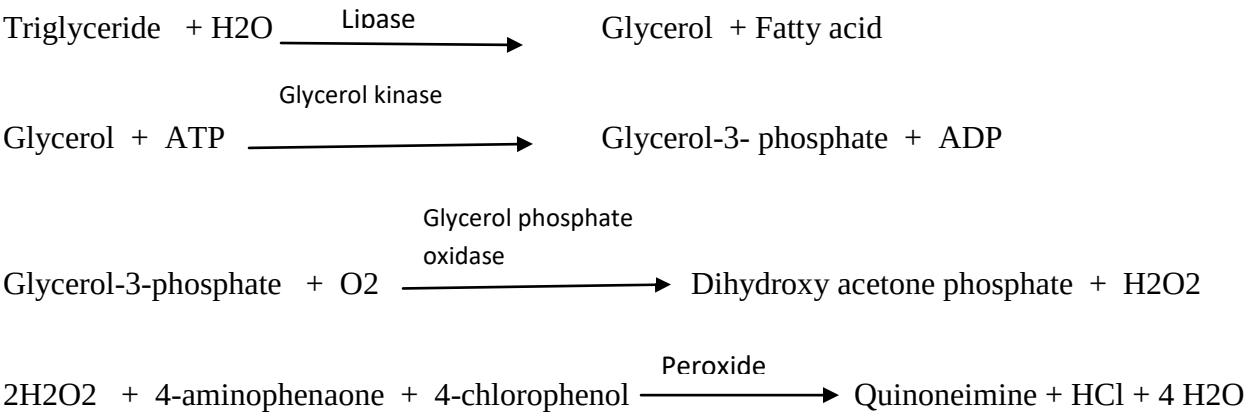
$$\text{Cholesterol concentration (mg/dl)} = \frac{\Delta A\text{sample}}{\Delta A\text{ standard}} \times \text{concentration of standard}$$

2.7.3 Estimation of serum triglyceride (TG) by enzymatic colorimetric method

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Dimension LR, Siemens Ltd., UK using reagents of Randox Laboratories Ltd., UK

Principle

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerol kinase and ATP.Glycerol-3-phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_20_2. In the presence of peroxidase, hydrogen peroxide oxidizes thechromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system.



Content (Reagent)	Concentration in the test
Pipe Buffer	40 mmol/l, pH 7.6
4-chlorophenol	5.5 mmol/l
Megnesium ions	17.5 mmol/l
ATP	1 mmol/l
Lipase	>150 U/ml
Glycerol-3-phosphate oxidase	1.5U/ml
Peroxidase	0.5U/ml

Table 2.3: Reagent used in enzymatic colorimetric method

Materials

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer

Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five 5µl sample and 200µl reagent were mixed and incubated at 37°C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of standard}$$

2.8. RNA Extraction from tissue by TRIzol reagent

After 8 weeks the rats were sacrificed and tissue were collected immediately and incubate in a ice cold condition.

Reagents required

- Chloroform
- Isopropyl alcohol
- 75% Ethanol
- RNase-free water

The TRIzol usage on RNA isolation

Added TRIzol reagent 1ml/ 100mg tissue was added to the tissue homogenization.TRIzol can inhibit the breakdown the RNA.Incubated at room temperature for 5 min.Added 200µl chloroform/1 ml TRIzol.Shaked the tubes vigorously by hand for 15 seconds. Again Incubatedat

room temperature for 3 min.Then Centrifuge 15 min at 4oC at less than 12,000 g (10000 rpm, r=65 mm).

Gravity force or g force (RCF) = (RPM ÷1000)2 ×1.118× r (here r = cm)

RPM = √[RCF÷ (r × 1.118×10¯6)] (here r = mm)

g= Relative centrifuge force

r = Rotational radius

RNA was in top (aqueous phase, about 60% volume).Transfered the RNA aqueous phase (top) to a fresh tube.Added 500µl isopropanol/ml TRIzol.Incubated 10 min at room temperature. Then Centrifuge 10 minutes at 40°C by less than 12000 g.RNA was in bottom as gel-like pellet.Removed and disposed the supernatant.Wash RNA pellet once with 75% ethanol by adding 75% ethanol 1 ml/ml TRIzol.Vortex and centrifuge 5 min at 40°C by less than 7500 g (8000 rpm, r=65 mm).The supernatant was discarded and saved RNA pellet (air-dry 5-10 min).Dissolved RNA in 50µl RNase-free water, Mixed with pipette.Incubated 10 min at 58ºC in waterbath. Finally stored at -32ºC.

2.9. cDNA synthesis: Reaction mixture for cDNA

Mix	X 10
Oligo dT	**5µl**
5X buffer	**20µl**
RNase inhibitor	**5µl**
dNTPs	**10µl**
Nuclease free water	**50µl**
RT (MMULV)	**5µl**
Total = 95 µl	

Table 2.4: cDNA preparation (Reaction volume 10µl)

The cDNA reaction mixture was taken in each micro-centrifuge tube and 0.5 µl of RNA was added for cDNA synthesis .Incubate the mixture at 42°C for 1 hr. Again incubate 70°C for 5 min and finally stored at -32ºC

2.10. PCR for mRNA expression: Reaction mixture for PCR

SL.No.	Mix	For GLUT-4, AMPKα-2 and GAPDH primer (1X)	For AMPKα-1 primer (1X)
01.	cDNA	1.0 µl	1.5 µl
02.	10x PCR buffer	1.0 µl	1.0 µl
03.	dNTPs	0.2 µl	0.2 µl
04.	$MgCl_2$	0.6 µl	0.6 µl
05.	Forward primer	0.3 µl	0.3 µl
06.	Revers primer	0.3 µl	0.3 µl
07.	Tag	0.1 µl	0.1 µl
08.	H_2O	6.5 µl	6.0 µl
	Total	10 µl	10µl

Table 2.5: PCR mix preparation (Reaction volume 10µl)

This mixture was took into PCR tube and transferred on thermocycler which performed 30 cycle. After polymerase chain reaction (PCR) this sample run in 3% agar gel and then finally identified our desired product expression by gel doct method.

2.11 Protein analysis by western bloting

2.11.1. Tissue Sample Preparation

Typically, samples were taken from tissues (i.e. muscle, adipose, and liver) of rat. These samples were cooled or frozen rapidly. They were homogenized using moter & pestle with RIPA (Radio immuno-precipitation assay) Lysis Buffer.

Reagent	Volume (1ml)
PMSF solution	20µl
Na-Orthovanadate	10µl
Phosphatase cocktail	10µl
Protease cocktail	10µl
1x RIPA lysis buffer	950µl

Table 2.6: Reagent complete RIPA lysis buffer

Figure 2.7 : Protein extraction using RIPA buffer

Procedure

About 200 mg tissue was isolated.1ml of RIPA Buffer was added into tissue sample. Sample was homogenized on ice bath with moter & pastel. Sample was centrifuged at 12,000 rpm for 20 minutes at 4°C.Supernatant was kept at -32°C until usage.

The prepared sample was then assayed for protein content using Bradford Protein Assay method, so that a constant amount of protein could be used for Western Blot.Protein concentration in tissue extracts were measured using BRADFORD Protein Assay Method.

Content(Bradford reagent)	Volume (200ml)
Coomarsie blue	100mg
Methanol	50 ml
Phosphoric acid	100 ml
dd water	50ml

Table 2.7: Component of Bradford reagent

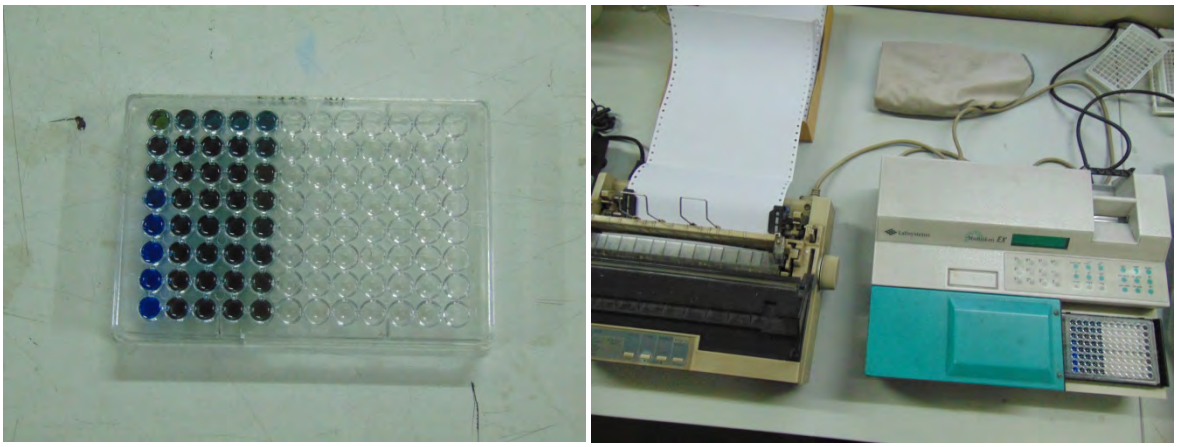


Figure 2.8: Protein concentration measurement

Denaturation of proteins for Polyacrymide gel electrophoresis

About 90µl sample was boiled at 95ºC for 5 minutes with 30µl of 4X Laemmli's buffer to denature the samples, this buffer contains dye, a sulfurous compound- typically P-mercaptoethanol and a detergent known as SDS (Sodium dodecyl sulfate). The boiling denatures the proteins, unfolding them completely. The SDS then surrounds the protein with a negative charge and the p mercaptoethanol prevents thereformations of disulfide bonds.

2.11.2 Gel Electrophoresis

The proteins of the sample are separated according to molecular weight using gel electrophoresis. Gels have various formulations depending on the lab, molecular weight of the proteins of interest. Polyacrylamide gels are most common. Since the proteins travel only in one dimension along the gel, samples are loaded side by side into wells formed in the gel. Proteins are separated by mass into bands within each lane formed under the wells. One lane is reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights.

2.11.2.1. SDS Polyacrylamide Gel Preparation

There are two sequential portion of gel with different concentrations of acrylamide, the top portion, called the stacking gel, is slightly acidic (ph 6.8) and has a low acrylamide concentration to make a porous gel. Under these conditions proteins separate poorly but form

thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (ph 8.8), and has a higher polyacrylamide content, which causes the gel to have narrower channels or pores. As a protein, concentrated into sharp bands by the stacking gel, travels through the separating gel, the narrower pores have a sieving effect, allowing smaller proteins to travel more easily and hence rapidly, than larger proteins.

2.11.2.1.1. 12 % separating or running gel preparation

40% Acrylamide/Bis (29:1)	6ml
1.5M tris.HCl (pH 8.8)	5ml
dd water	8.6ml
20% SDS (Sodium Dodecyl Sulphate)	200µl
10% APS (Amonium per Sulphate)	200µl
TEMED (Tetramethylethylenediamine)	20µl

Table 2.8: Chemical required for running gel (20ml)

1.5 M & 0.5 M Trizma HCl Preparation for 100ml (M.W= 157)

1 mole Trizma HCl = 157 g Trizma HCl

Now,

To make 1 M Trizma HCl solution in 1000ml dd water Trizma HCl need = 157g

To make 1.5 M -- -- -- -- 100ml -- -- -- -- -- = (157×1.5×100) ÷1000

= 23.55g

Procedure: 23.55g Trizma HCl measure using electric balance and take it into a beaker. Add 50 ml dd water and put the beaker on the stirrer. After dissolving the reagent set the pH 8.8 using 3N NaOH. After setting the pH solution take into a 100ml measuring cylinder and add dd water up to 100ml.1.5M Trizma HCl solution have prepared. It preserve at room temperature.

Caution

- Solution must contain 8.8 pH.

Again,

To make 1 M Trizma HCl solution in 1000ml dd water Trizma HCl need = 157g

To make 0.5 M -- -- -- -- 100ml -- -- -- -- -- = (157×0.5×100) ÷1000

=7.85g

Procedure: 7.85g Trizma HCl measure using electric balance and take it into a beaker. Add 50 ml dd water and put the beaker on the stirrer. After dissolving the reagent set the pH 6.8 using 3N NaOH. After setting the pH solution take into a 100ml measuring cylinder and add dd water up to 100ml.0.5M Trizma HCl solution have prepared. It preserve at room temperature.

Caution

- Solution must contain 6.8 pH.

3 N sodium Hydroxide preparation for pH control of trizma HCl solution (100ml)

To make 1 N NaOH solution in 1000ml dd water NaOH need = 40g

To make 3 N -- -- -- -- 100ml -- -- -- -- -- = (40×3×100) ÷1000

=12g

[Normality consider equivalent wt of solute]

Procedure: 12g NaOH take into a beaker and add dd 50ml water.The beaker put on stirrer. After dissolving the reagent, solution transfer into 100ml measuring cylinder and add dd water up to 100 ml. 3N NaOH solution have prepared. It preserve at room temperature.

10% Ammonium per Sulphate preparation for 10ml

100 ml dd water need = 10g APS

10ml -- -- -- = 1g APS

Procedure: 1g APS take into blue falcon tube (25ml). Add 5ml dd water and shake. Finally add dd water up to 10 ml.

Caution:

- It should preserve – 4°C

Procedure for 12% running gel preparation: All reagents take into a yellow falcon tube according to the chart but be careful about TEMED. Add TEMED just before loading the gel because it solidified the gel.

2.11.2.1.2. 4% stacking gel preparation

40% Acrylamide/Bis (29:1)	0.5ml
0.5M tris.HCl (pH 6.8)	1.25 ml
dd water	3.1ml
20% SDS (Sodium Dod ecyl Sulphate)	25µl
10% APS (Amonium per Sulphate)	50µl
TEMED (Tetramethylethylenediamine)	5µl

Table 2.9: Chemical required for stacking gel (5ml)

Procedure for 4% staking gel preparation: All reagents take into a blue folicon tube according to the chart but be careful about TEMED. Add TEMED just before loading the gel because it solidified the gel.

2.11.2.2. Electrophoresis

10X running buffer preparation (Stock)

Trizma Base	**7.6g**
Glicine	36g
dd water	Up to 250 ml

Table 2.10: Reagent required for 10X running buffer (250ml)

Procedure: Trizma base and glycine measured in a electric balance and take into a beaker . Add 100ml water and put on stirrer for dissolving the solute. After dissolve solution take into measuring cylinder and add water up to 250ml.

1X running buffer Preparation (Working)

Stock running buffer	**100ml**
20% SDS	5ml
dd water	Up to 1000ml

Table 2.11: Reagent required for 1X running buffer (1000ml)

Procedure: 100ml stock running buffer take into measuring cylinder. Then 5 ml 20% SDS add to the cylinder and finally add dd water up to 1000ml.Now 10X running buffer have prepared.

1X PBS (Phosphate buffer saline) preparation

NaCl	**8g**
KCl	0.2g
Na2HPO4	1.44g
K2HPO4	0.24g
dd water	Up to 1000 ml

Table 2.12: Reagent required for 1X PBS (1000ml)

Procedure: All reagent measure by electric balance according to their required weight. Take a small (500 ml) beaker and take 200-250ml dd water. Add all reagent one by one into the beaker. The beaker put on electric starrer. After dissolving all reagents set pH 7.4.Then solution take into 1000ml measuring cylinder and add water up to 1000ml. After prepare 1X PBS autoclave have done. It should preserve at – 4°C.

Caution:

- 1X PBS must contain 7.4 pH
- Autoclave should need
- It should preserve – 4°C

The comb was placed into the stacking gel until the gel was aliquoted. The top electrolyte compartment was filled with running buffer. The leaks were checked from the top into the bottom compartment. If there were no leaks then the bottom compartment was filled.The comb was removed and clamped the gel to the electrophoretic apparatus. With a plastic Pasteur pipette, thoroughly rinsed each well in the stacking gel with running buffer. Samples were injected by using a micropipette carefully.12µl of Protein Marker or Ladder was injected one or two wells, preferably in an asymmetric position, to allow the front and back of the gel to be identified later. The contents of each well were recorded carefully.Electrophoresis was carried out for about 3hrs at 40-120 volts, voltage was increased when dyes of all the lanes were in a line.Always the

power was turned down and unplugged the wires from the power supply before removing the cover.

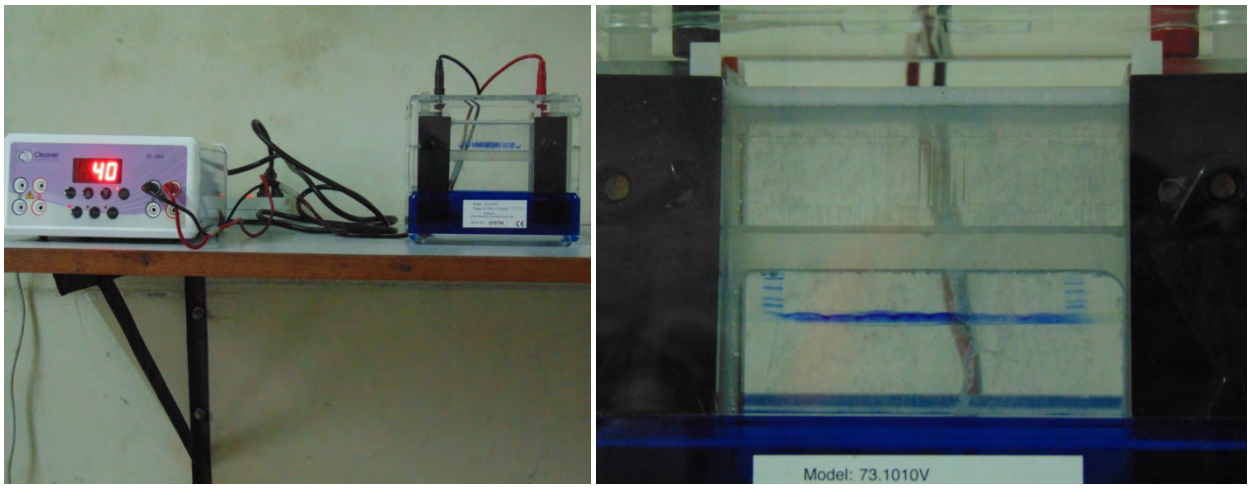


Figure 2.9: Polyacrylamide Gel Electrophorosis

2.11.2.3 Protein transfer to PVDF membrane

Transfer buffer preparation:

10X running buffer (Stock)	100ml
dd water	700ml
Methanol	200ml

Table2.13: Reagent required for transfer buffer (1000ml)

Procedure: 100ml 10X running buffer (stock) take into a measuring cylinder. Add 700ml dd water and finally add 200ml methanol to make 1000ml final volume. It is prepare for use.

Caution

- Transfer buffer should preserve at – 4°C.

The polyacrylamide gel is good for separating of protein but not suitable for the staining and the further detecting. Materials were wet in Protein transfer buffer.

The following order was stacked:

Case (black side)

Sponge

Whatman (Chromatography) paper

Gel

PVDF Membrane

Whatman (Chromatography) paper

Sponge

Case (red side)

The complete case was placed in the transfer apparatus at 400 mA electricity for 2 hours to transfer proteins from gel to PVDF Membrane.

Blocking: Membrane was blocked for 1 hour in 3ml blocking solution containing 5%BSA for hosphorproteins and 5% non-fat milk for total proteins in 1X PBS with continuous agitation at room temperature.

Primary antibody incubation: After 1 hour, blocking solution was discarded. The membrane was incubated with primary antibody (anti- p-AMPKa ½ (Thr 172) at 1:75 dilution in blocking solution at 4°C for overnight with continuous agitation.

Washing: After discarding the primary antibody solution the membrane was washed 3 times (5 minutes each time) with lxPBS to clean the membrane from unbound antibody.

Secondary antibody incubation: Immediately the membrane was incubated with secondary antibody named by goat anti- rabbitIgG-HRP (sc: 2054)[1:100 dilution].The PVDF membrane and secondary antibody solution were sealed in a small plastic bag. Incubation was performed for 1 hour at room temperature on a rotator device (RPM: 12).

Figure 2.10: Incubation performed on rotator

Washing: Blocking solution with secondary antibody was discarded. The membrane was washed 5 times (5minutes each time) with lxPBS .

Enhanced chemiluminescence (ECL) solution: The PVDF membrane was incubated with ECL solution for 5minutes. The autoradiography film was exposed to PVDF membrane in X-ray cassette for a certain time in a dark room. Then the film was developed manually using developing and fixation solution.The desired band was identified with comparison of the known molecular weight protein marker.

Stripping for total protein

Reagent	Volume (300ml)
20% SDS	3ml
Glycin	1.325mg
dd water	Up to 300ml

Table 2.14:Reagent required for Stripping solution

Slowly added HCl in 200ml striping solution to obtain pH 2.6.Then added dd water to make volume 300ml and preserved at 4ºC.PVDF membrane washed with this stripping solution for 5 minute.

2.12.Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 12 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean±SD, number (percentage) as appropriate. The statistical difference between two groups was assessed by unpaired‘t’ test and paired ‘t’ tests. A two-tailed p value of <0.05 was considered statistically significant.

Chapter Three

Results

3.1 Effect of Oyster Mushroom on the body weight of type 2 diabetic model rats

Changes in body weight in different groups of rat have been depicted in table 3.1. Initial body weight (g) were 193±17, 196±10 and 201±26 in water control diabetic rats, Gliclazide treated and Mushroom treated diabetic model rats respectively.The body weights of rats under the study were recorded every week and the results have been presented in table 3.1.Although the body weight in different weeks among all the three groups of the rats have shown some changes, however, when the body weight was compared within the group by one way ANOVA, no significant difference have been found in any of the groups.

Table 3.1: Body weight of different groups of type 2 diabetic model rats during the experimental periods.

group	Body weight (Gram) from 0 day to 56 day (M±SD)								
	0 day	7 day	14 day	21 day	28 day	35 day	42 day	48 day	56 day
WC (n=6)	193±17	226±16	233±19	237±18	245±23	240±26	238±34 (23%)	271±51	271±50 (40%)
GT (n=6)	196±10	216±10	230±6	248±13	251±14	260±15	266±12 (35%)	270±17	280±17 (42%)
MT (n=6)	201±26	227±29	237±30	251±33	253±33	265±37	260±38 (29%)	298±7	301±2 (49%)
One way ANOVA									
WC Vs GT	1.000	1.000	1.000	1.000	1.000	0.705	0.388	1.000	1.000
WC Vs MT	1.000	1.000	1.000	0.928	1.000	0.428	0.715	1.000	0.839
GT Vs MT	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.936	1.000

Group WC, GT and MT represent water control diabetic model rat, Gliclazide treated diabetic rat and Mushroom treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA.

Figure 3.1 shows thatthe body weight of diabetic model rats increased in every week of the experimental period. When percentage values regarding body weight change were compared it was found that on day 42nd day increase in body weight was 23%, 35% and 29% in WC, GT and MT groups respectively. Similarly on 56thday percentage of body weight increased by 40%, 42% and 49% in WC, GT and MT respectively (Table 3.1). Increased in body weight was maximum in Mushroom treated diabetic rats than the water control and gliclazide treated rats.

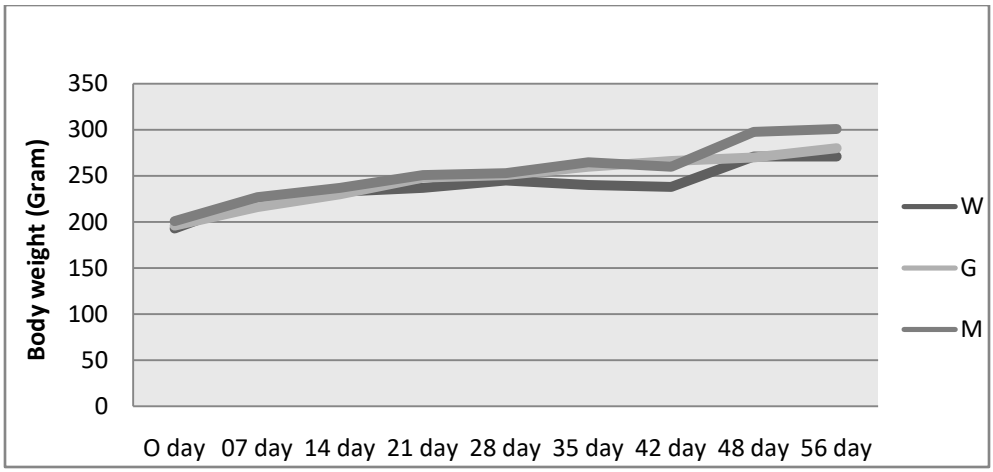


Figure 3.1: Body weight of diabetic model rat from 0-56 days. Here W,G and M represent Water control, Gliclazide treated and Mushroom treated diabetic rats.

3.2 Effect of Oyster Mushroom on serum glucose level of type 2 diabetic model rats

To evaluate the effect of Oyster Mushroom on glucose metabolism, fasting serum glucose levels of different experimental groups were measured.As it is seen from the table 3.2 at baseline, fasting serum glucose level was 8.52±2.49 (mmol/L) in mushroom treated groups (group-3). During the 42 day study period there was a gradual decrease in fasting serum glucose level of type 2 diabetic model rats.On day 42 fasting serum glucose was 6.88±1.17 (mmol/L) and at day 56 it was 5.62 ± 2.02 (mmol/L) respectively.Serum glucose level decreased by 33% within 56 days in comparison to 0 day values.So serum glucose level was found to be decreased gradually but the decreasewas non-statistically significant.It is also evident from the table 3.2 that the serum glucose level ofwater control diabetic rat (group-1) on 42 day of the study period also decreased nonsignificantly. However, this low level of glucose did not persist for a longer period. As it is depicted on the table glucose level started to increase and it was 4.66 ±1.78 mmol/L compared to 3.84 ±0.611mmol/L on day 42.In Gliclazide treated group (group-2) fasting glucose concentration were 10.01±3.44, 10.48 ±2.66 and 6.70 ±2.82 on 0 day, 42 day and 56 day respectively.So Glicalzide treated group showed a 33% decrease in serum glucose levelwhen it was compared with initial dayhowever, this decrease was also non-significant.

Table 3.2: Fasting serum glucose level in different groups of type 2 diabetic model rats 0 day, 42 day and 56 day of the experiment.

Group	Fasting serum glucose, mmol/L (M±SD)		
	0 day	42 day	56 day
WC(n=6)	7.18 ±1.80(100%)	3.84 ±0.611(53.48%)	4.66 ±1.78(65 %)
GT(n=6)	10.01 ±3.44(100%)	10.48 ±2.66(100%)	6.70 ±2.82(67%)
MT(n=6)	8.52 ±2.49(100%)	6.88 ±1.17(81%)	5.62 ±2.02(67%)
One way ANOVA			
WT Vs GT	0.255	0.010	1.000
WT Vs MT	1.000	0.220	1.000
GTVs MT	1.000	0.127	1.000
P- Values (Paired sample t test)			
Group	O day Vs 42 day	0 day Vs 56 day	
WC	0.111	0.027	
GT	0.359	**0.084**	
MT	0.917	**0.092**	

Group WC, GT and MT represent water control diabetic model rat, Gliclazide treated diabetic rat and Mushroom treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

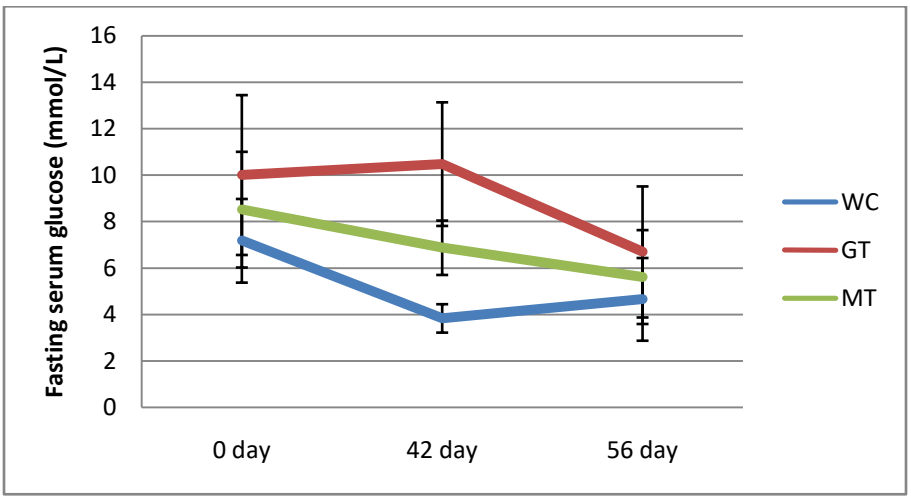


Figure 3.2: Fasting blood glucose level of different groups of type two diabetic model rats of 0 day, 42 day and 56 day

3.3 Effect of Oyster Mushroom on serum triglyceride and total cholesterol level of STZ-induced type 2 diabetic model rats

Chronic effect of Oyster Mushroom on serum cholesterol level is present in Table 3.3.In mid of the experiment triglyceride level of mushroom treated rats was increased but at the end of the experimental period it was reduced by 4% from the initial day. Mushroom treated rats increased total cholesterol level 63% at 42th day but at end of the experiment it became 32%. Since there is a tendency of lowering the harmful lipids, so there is a possibility that after long time treatment mushroom could reduce triglyceride level as well as total cholesterol. As expected, the standard drug Gliclazide treated group showed a decreased level of both triglyceride 37% and total cholesterol level 17% at the end of the study period. Water Diabetic control rats showed a 11% decrease in triglyceride level & a 42% increase in total cholesterol level on 56th day.

Table 3.3 :Effect of Oyster Mushroom on lipid profiles of T2DM model rats

Group	Triglycerides level (mg/L)			Total cholesterol level (mg/L)		
	0 day	42 day	56day	0 day	42 day	56 day
WC	84±8 (100%)	80±16 (95%)	75±20 (89%)	56±9 (100%)	80±22 (142%)	80±11 (142%)
GT	90±43 (100%)	66±14 (73%)	57±15 (63%)	73±25 (100%)	68±12 (93%)	61±16 (83%)
MT	64±17 (100%)	85±24 (132%)	62±7 (96%)	49±5 (100%)	80±25 (163%)	65±3 (132%)

P-Values (Paired Samples T test)				
Group	0 day vs. 42 day	0 day vs. 56 day	0 day vs. 42 day	0 day vs. 56 day
WC	0.801	0.504	0.368	0.000
GT	0.598	0.247	0.944	0.309
MT	0.029	0.813	0.155	0.059

Group WC, GT and MT represent water control diabetic model rat, Gliclazide treated diabetic rat and Mushroom treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD).Statistical comparison between groups was performed using paired sample t test.

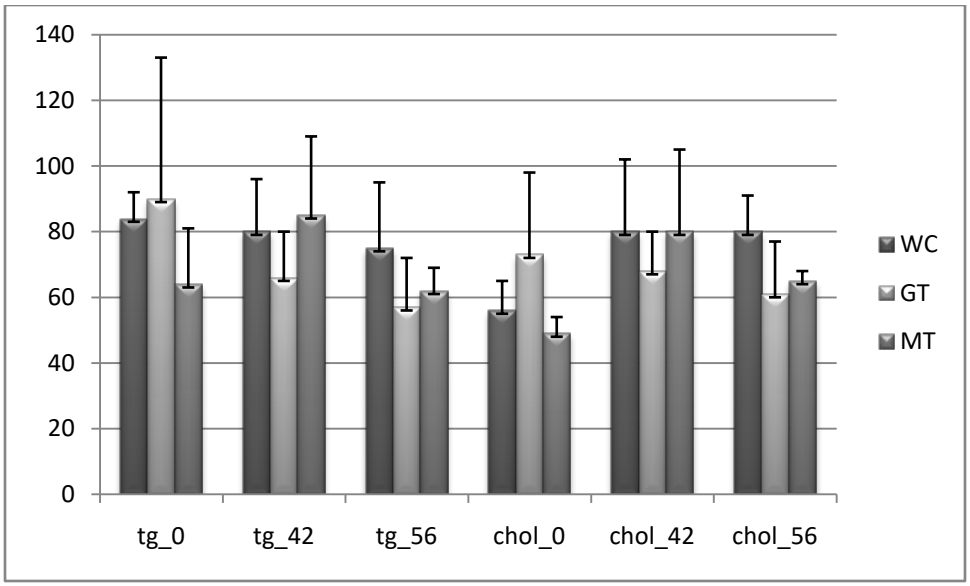


Figure 3.3: Effect of Oyster Mushroom on lipid profiles of T2DM model rats

3.4. Effect of Oyster Mushroom on the p-AMPK protein in muscle and adipose tissue of type 2 diabetic model rats

In figure (3.4-3.5) p-AMPK from muscle and adipose tissue have been detected from non diabetic, diabetic, mushroom treated and gliclazide treated rats have been detected between the marker protein of 85KDa and 50KDa, p-AMPK seems to be decreased in STZ induced diabetic model rats (water contror) compare to non diabetic rats.But in case of mushroom treated diabetic rats, p-AMPK have increased compare to control rats (A).Besides, in case of adipose tissue (B), p-AMPK seems to decrease compare to non diabetic rats. Mushroom treated rats has increased its p-AMPK protein better than the gliclazide treated rats.

Although equal amounts of protein (400µg) was loaded in electrophoresis but the membrane was also bloted for a house-keeping protein β-actin which shows the equilibrium of protein loading. But in case of mushroom treated diabetic rats β-actin has decreased compare to non-diabetic, diabetic, gliclazide treated rats (3.4-3.5).

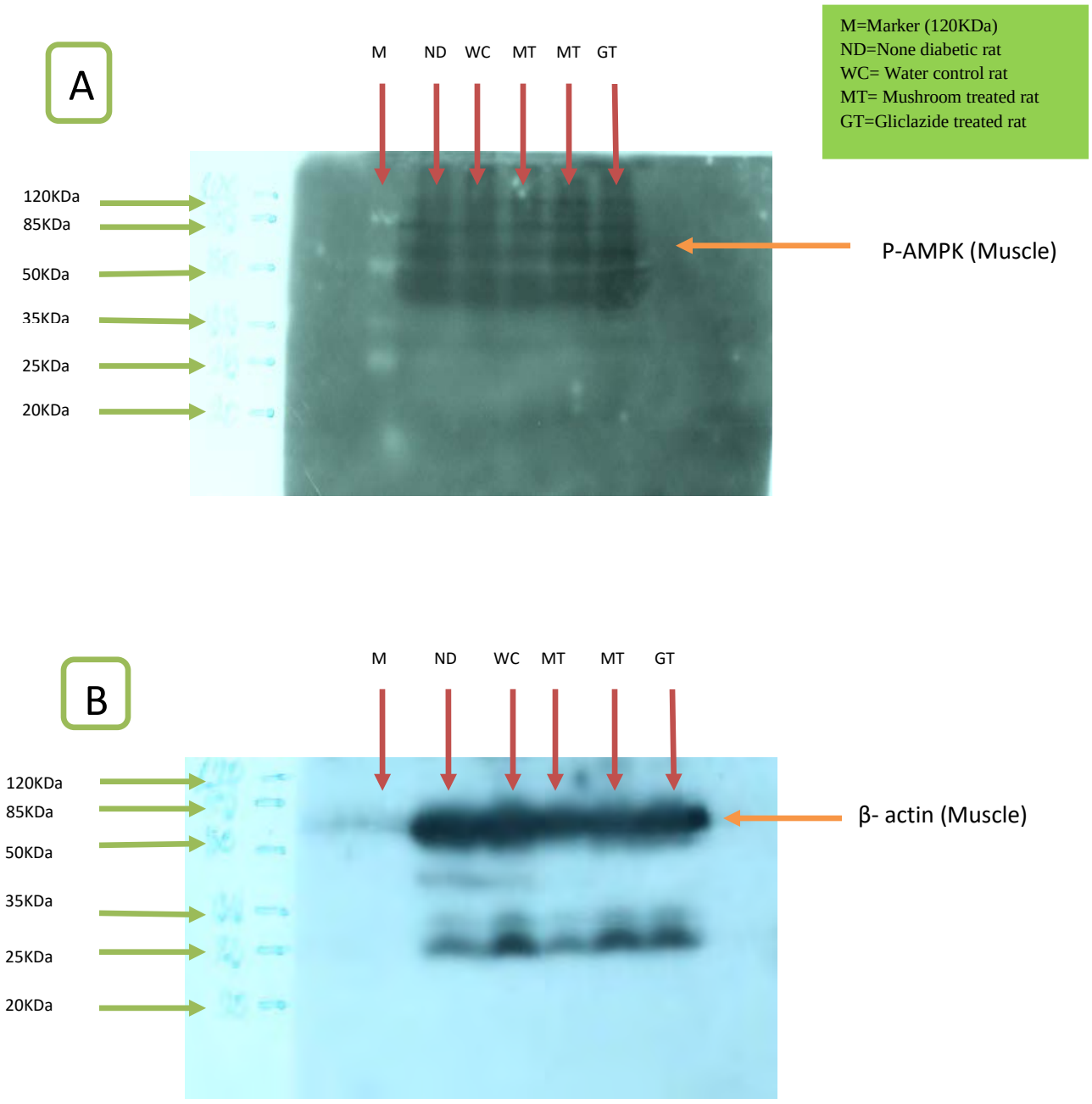


Figure 3.4: AMPK protein expression of muscle tissue.'A' indicates 500µg of Protein from muscle tissues of rats were analyzed using western blotting for p-AMPK. ''B' indicates protein from muscle tissues of rats were analyzed using western blotting for β-actin.

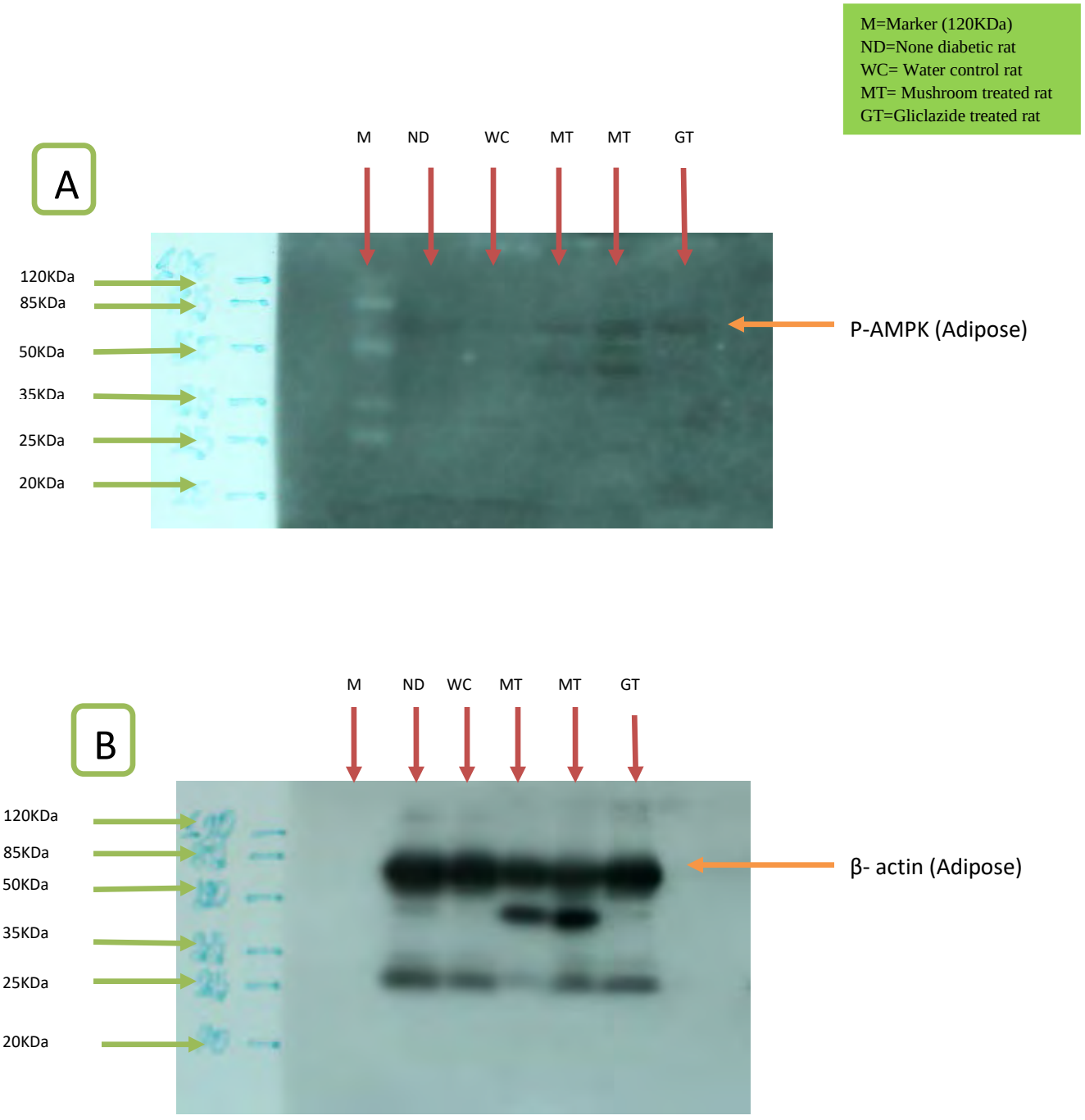


Figure 3.5: AMPK protein expression of adipose tissue. 'A' indicates 200µg of Protein from adipose tissues of rats were analyzed using western blotting for p-AMPK. ''B' indicates protein from adipose tissues of rats were analyzed using western blotting for β-actin.

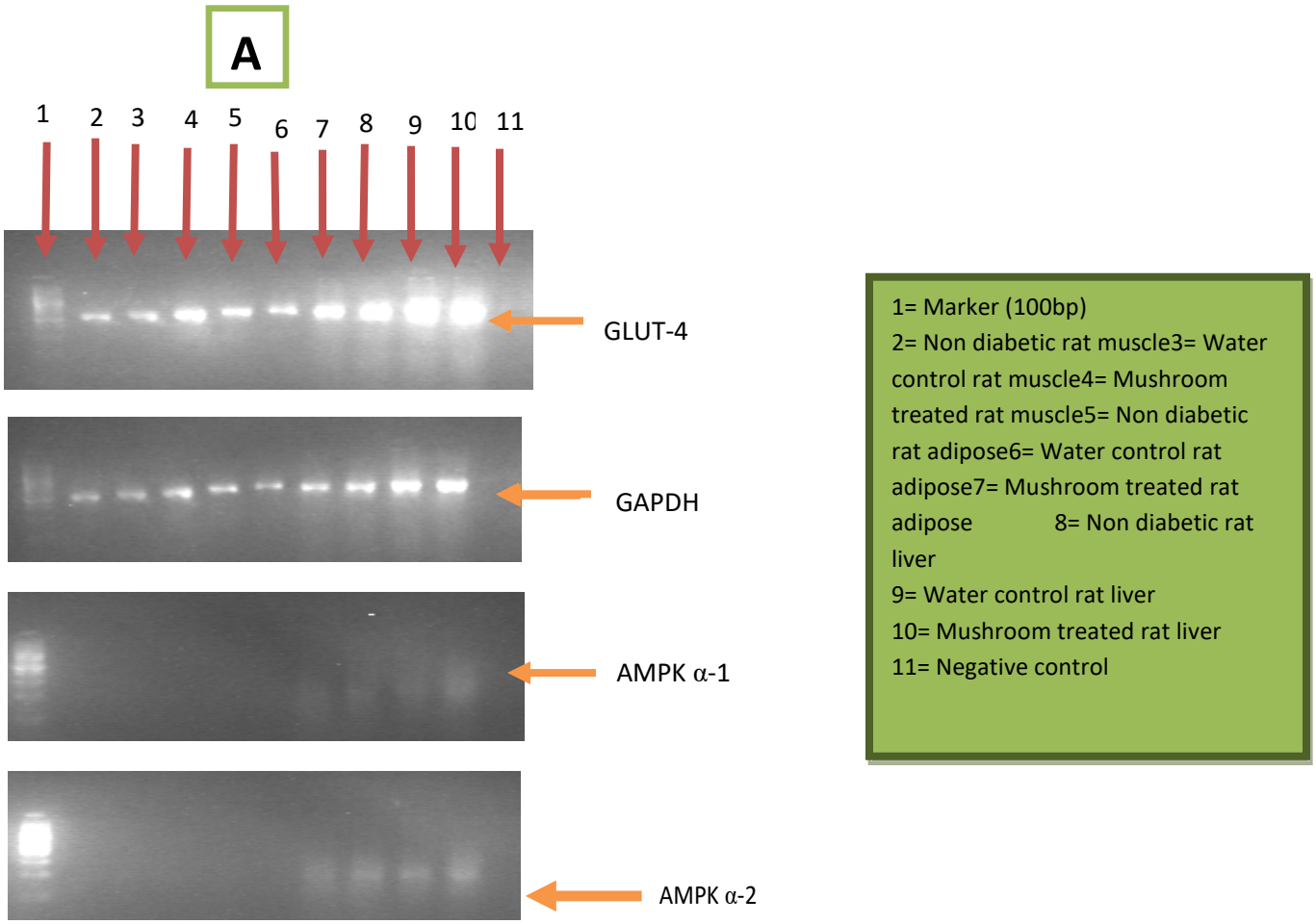


Figure 3.6: Expression of Glut 4, GAPDH, and AMPK subunit isoform (α1,α2)in type 2 diabetic model rat.

3.5. Effect of Oyster Mushroom on mRNA expression in type 2 diabetic rats

In figure 3.5 shows expression of GLUT4 (Glucose transporter type 4), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase),AMPKα1 & α2 (AMP-activated protein kinase) genes of different samples (Muscle, Adipose, Liver). Although in figure 3.5 shows the increased expression of housekeeping gene GAPDH (about 3-4 fold) but the increment of GLUT-4 is much more (about 20 fold) compared to GAPDHin muscle , adipose and liver tissue of mushroom treated diabetic model rats. AMPKα-1 and AMPKα-2 have not shown in muscle tissue, it poorly expressed in adipose and liver tissue but mushroom treated rats shows higher expression.Therefore the expression shows that Oyster Mushroom has effect on gene expression of GLUT-4, AMPKα-1 and AMPKα-2.

Chapter Four

Discussion & Conclusion

4.1. Discussion

Diabetes mellitus is being increasingly recognized as a serious global health problem and is frequently associated with co-morbid distress, contributing double burden for the individual and the society. It is the fourth or fifth leading cause of death in most high-income countries [43]. It is a group of diseases characterized by high levels of blood glucose resulting from defects in insulin production, insulin action and both, and is associated with serious complications and premature death [44]. Diabetes is a form of chronic illness usually requiring glycemic monitoring, self-management education and support to prevent acute complications and to reduce the risk of long term complications [45]. Previous animal study showed that, diabetes is accompanied by loss of weight [46].The body weight of untreated diabetic rat is reduced as compared to the normal rats. The blood glucose levels of STZ-induced diabetic rats is higher when compare to the normal rats [47]. Hypercholesterolaemia and hypertriglyceridaemia have been reported to occur in STZ diabetic rats [48].

In our study with STZ-induced type 2 diabetic rats, there was a nonsignificant increase in body weight of each group of rats (Water control, Mushroom treated and Glibenclamide treated) throughout the experimental period. However, when body weight was compared between different study groups no significant difference was found. Our experiment shows that White Oyster Mushroom when supplemented with rat feed, it reduced blood glucose level compared to other groups. It was also found that diadetic control group showed low blood glucose level on the 42nd day of the experimental period. It may be explained by the fact that on 42 day of the experimental period body weight of this group was less compared to GT & MT groups which may be due to less intake of food. The less intake of food may be due to some toxic effect which may result in the manifestation of low glucose level of WC group on 42 day of study period. The glucose level of diabetic water control group rose at the end of the study period. We have also found that White Oyster Mushroom showed anti hypertriglyceridaemic effect because over expression of AMPK α2 subunit in the liver decreases plasma TG levels [49] but it exhibited hypercholesterolaemia of STZ-induced type 2 diabetic rats.

AMP-activated protein kinase (AMPK) signaling pathway induce cellular resistance to glucose deprivation, ischemia, hypoxia, oxidative and senescent stress. AMPK plays a critical role in metabolism and stress-associated cellular processes. Studies have suggested that AMPK signaling is involved in regulating efficient energy consumption, by mediating metabolic homeostasis, enhanced stress resistance and qualified cellular housekeeping [50]. Activation of AMP-activated protein kinase (AMPK) inhibits hepatic fatty acid synthesis [51,52]. In all cases, activation of AMPK requires phosphorylation of T-172 by an upstream protein kinase. Once activated, AMPK increases cellular energy supply by switching on ATP-generating pathways and decreases energy demand by switching off ATP-utilizing pathways. Thus, AMPK serves as a "fuel gauge" that responds to fluctuations in cellular energy level, as well as, to the levels of specific extracellular nutrients such as glucose and fatty acids and hormones [52]. AMPK may be activated by contraction of muscle or 5 aminoimidazole-4 carboxamide-1-b-D-ribofuranoside (AICAR), leading to increase in glucose uptake. Insulin enhances the glucose uptake by activating the insulin receptor. The possibility is that AMPK may regulate glucose transporter activity.IH-901 stimulate glucose uptake with parallel increase in GLUT4 translocation to the plasma membrane possibly via activating AMPK pathways [49]. GLUT4 is a good candidate to regulate glucose sensing due to its localization in regions associated with glucose sensing, co-expression with the insulin receptor [18]. The principal glucose transporter protein that mediates this uptake is one isoform (Gene name, SLC2A4; protein name: GLUT4) of a family of sugar transporter proteins containing 12-transmembrane domains. The GLUT4 glucose transporter is thus a major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis [53]. It exists only in skeletal muscle and adipose tissues, which are responsible for 50% to 80% of glucose transportation in the body. It has been reported that the skeletal expression of GLUT4 in type 2 diabetes patients is significantly reduced, indicating that such patients have less capability to process glucose. The inhibition of GLUT4 translocation is one of the most important reasons for poor glucose transportation in the body [54]. Diabetes in animals and patients, and hyperglycemia in cells, all decrease the activity of the key glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in cell types that develop intracellular hyperglycemia [55].

In this study it has been observed that the effect of Oyster Mushroom on the expression of GLUT-4 and AMPK. After 8 weeks of mushroom treated diabetic model rats has shown increased expression of GLUT-4 and AMPK gene through cDNA and rt-PCR experiments. GAPDH gene was considered as control since it is known as house keeping gene as expressed consistency. It has been found that GAPDH gene expression was not changed in diabetic and mushroom treated diabetic model rats but expression of GLUT-4 and AMPK decreases in diabetic model rats which reproduce by mushroom treated rats. Therefore, mushroom could play role for the controlling of blood glucose level through expression of GLUT-4 and AMPK proteins.

In our study, gel electrophoresis was done to identify the gene expression of GLUT4, AMPK (α1& α2) subunit with different tissue samples cDNA (Muscle, Adipose, Liver) of STZ induced Type 2 diabetic model rats. It was found that White Oyester Mushroom treated rats gave the higher expression of all four genes than the control group of diabetic and non diabetic rats (Figure 3.5 A). When we compared this with drug treated rat to identify GLUT4 and AMPK α2 gene expression then we found higher expression of interested gene than that in the non diabetic, water control and drug treated rats. This means that White Oyster Mushroom increases glucose transporter protein (GLUT4) production and removes glucose from the circulation. Thus increases the activity of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The higher expression of AMPK (α1 & α2) subunits indicates more activations of AMPK signaling pathway.

The major objective of this study was to investigate the effect of Oyster Mushroom on the activation of AMPK signaling pathway. This study has documented that activated AMPK which is reduced in STZ-induced diabetic rats, has improved after 8 weeks treatment with Oyster Mushroom. This study has also shown that muscle tissue produces more p-AMPK compared to adipose tissue of Long-Evan's rat. When we have looked about the total AMPK status using western blot analysis of tissue proteins, it has been shown that expression of AMPK was reduced in STZ-induced diabetic rats where the mushroom treated rats seems to have improved the AMPK levels. AMPK activation results increased glucose uptake in skeletal muscle and decreased hepatic glucose production. Although Oyster Mushroom has not increased phosphorylation of AMPK but increased expression of AMPK and this may help to regulate

glucose metabolism through increased expression of other genes. GLUT-4 expression of mushroom treated diabetic rats seems to increases when compared to control rats where the involvement of GLUT-4 decreases.

4.2. Conclusion

From the results of the study it may be concluded that a) Oyster Mushroom when supplemented with normal feed can reduce hyperglycemia and hypertriglyceridemia in type 2 diabetic model rats b) Expression of GLUT-4 of mushroom treated diabetic rats increased as seen in result and thus it circular blood glucose level c) The improvement of glycemic status of Oyster Mushroom could be explained through partial activation of AMPK, and d) Therefore, Oyster Mushroom may function as metabolic regulator through activation of proteins in insulin signaling pathway.

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