Proteomic Profiling Of L6 Cell Lines to Identify Proteins That Can Serve As Potential Targets for Therapeutic Intervention of Type 2 Diabetes

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Abstract: - Type 2 Diabetes (T2D) is a metabolic disease characterized by abnormally high levels of sugar (glucose) in the blood (Hyperglycemia) which affects glucose homeostasis. It is caused due to the inability of the target cells to respond to insulin. Proteomics is the large-scale study of protein with their structures, functions, and information coded in a cell which is expressed and regulated at the protein level to achieve the function of an organism. Protein expression during the exposure of the cells to different glucose concentrations may alter and can give vital information about the pathogenesis of T2D. Study of underlying molecular mechanisms that lead to the development of T2D is important for the prevention and treatment of the disease the objective here is to study the effect of different glucose concentrations on proteomic profile of L6 cell lines. For this L6 skeletal muscle cells were exposed to different glucose (G) concentrations (0mM, and 25mM) for 48 hours. Total cell protein was extracted and protein profile was studied using SDS-PAGE. The identified proteins may provide information about disease pathophysiology and can serve as potential targets for therapeutic intervention of T2D.

Key Words: — ***Type 2 Diabetes, Glucose homeostasis, Proteomic profile, Cell protein.***

I. INTRODUCTION

The inability to properly utilize and/or store energy is the characteristic feature of metabolic disorders [1]. These may be inherited or acquired, the latter being more common and significant. Since these disorders affect the energy production or damage tissues critical for survival, these are clinically very important [2]. Diabetes is one of the most prominent metabolic disorders.

Hyperglycemia is the condition of high blood sugar level [3]. People with diabetes are affected by this condition. After our meals, glucose in the blood increases which is why the hormone insulin is released from the pancreas [4]. It prompts the liver to process the metabolism of glucose which causes the sugar level in the blood to reduce to normal level [5]. Sugar level of the blood remains high in a diabetic person. There are three reasons to explain this [6]:

- Insulin is not produced in proper quantity.
- Insulin is not being made at all.
- Insulin is not effective.

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Two of the most common types of diabetes are:-

- Type-1 diabetes: - It is an autoimmune disease. In this disease the pancreas are not able to produce insulin as the immune system of the patient attacks them.
- Type-2 diabetes: - It is a disease in which either the body resists insulin or there is a defect in the secretion of insulin.

L6 cell line of the rat and C2C12 cell line of the mouse are the two cell lines which are used to study the effect of glucose's high as well as low concentration [7]. In order for these cells to differentiate, once the cells become confluent, they are shifted from a media rich in serum to that of the media less rich [8]. Therefore, these cells differentiate from myoblasts to myotubes. To study the proliferation and differentiation of the muscle cells these myoblasts act as a good tool. Cultured myoblasts shows all the characteristics of muscle myogenesis which basically includes migration and proliferation, formation of myotubes and contraction [9]. Hence in this study we have used L-6 muscle cells to determine the glucose uptake activity of the cells and to identify the different proteins [9]. By studying the whole proteome we will be able to compare the expression of the protein in the normal and

diseased condition and thus will be able to understand the underlying mechanism of the disease that is Diabetes (type-2) [10].

II. METHODOLOGY

A. Cell Culture

Skeletal muscle cells of the rat (L6 cell line) were procured from NCCS, Pune, India. The maintenance and the growth of the cells were done in monolayer culture in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10mg ml-1 penicillin, 100mg ml-1 streptomycin in a condition of 5% CO2 at 37°C until they become 80-90% confluent.

B. Testing Of Cell Viability and Counting With the Help of Trypan Blue Exclusion

To know the number of living cells present in the cell suspension, trypan blue test was done. The main principle of this method is that the live cells contain cell membranes that are intact and therefore can exclude dyes like trypan blue, propidium, etc. The dead cells do not have intact cell membranes [11]. When the dye is being mixed with the cell suspension, it will either take up the dye or will exclude the dye. After mixing, if we will examine the cell suspension under microscope, the living or viable cell will be having a clear cytoplasm but the nonviable one will be having blue cytoplasm [11].

C. Differentiation of L6 Myoblasts into Myotubes

Rat L6 skeletal muscle cells were grown in DMEM supplemented with 10% FBS, 10µg ml-1 penicillin, 100 µg ml-1 streptomycin in the presence of 5% CO2 at 37°C until they become 80-90% confluent. For a 6 well tissue culture plate the seeding density is 0.3 × 105 cells per well. To trigger the differentiation of L6 myoblasts into myotubes, cells were cultured in glucose free DMEM (D-5030 Sigma Aldrich) containing 2% FBS (RM1112; Himedia) (differentiation medium, DM) for 7-8 days with medium change after 48 hours. Within 25-37 hours of incubation, the fusion of myoblasts starts. After around 48 hours, small myotubes were clearly seen. After 72-96 hours the small myotubes become large as well as mature.

The experiment was performed by inoculation of the cell suspension in six-well plate and incubated at 37°C for 24 hours. The cells were exposed to DM containing different glucose concentration. The different glucose concentrations were:

- *0mM(G)*
- *25mM(G)*

Cells were exposed to glucose free DM containing different glucose concentration for 48 hrs. The experiment was performed in triplicates. The seeding density per well in 6 well plate is 3 lakhs so the total cells needed per plate will be 18 lakhs after confluence. For the counting of the cells the Trypan blue dye exclusion test was performed.

D. Protein Extraction

The cells were trypsinized and centrifuged at 1200 rpm for five minutes. The pellet obtained was washed with PBS and 0.2 ml protein extraction buffer was added. The mixture was incubated on ice with tapping at every ten minutes. After one hour, centrifugation at 10,000 rpm was done for eighteen minutes at 4˚C. The supernatant was collected.

E. Protein Estimation and SDS-PAGE

Bradford assay is done to find out the total concentration of a protein in a sample. This method gives the measurement of the presence of the basic amino acid residues i.e. lysine, histidine and arginine since these contributes to the protein-dye complex formation. Absorbance was taken at 595nm. coomassie. The concentration of a protein in a test sample is determined by comparing the result to that of a protein standard. As standard, different proteins can be used but the most common and widely used protein is Bovine Serum Albumin (BSA). Lastly SDS-PAGE was done.

III. RESULTS

A. Cell Culture

2×105 cells were seeded in T-25 culture flask. After 72 hrs of seeding L6 cell lines showed confluency of about 90%. .

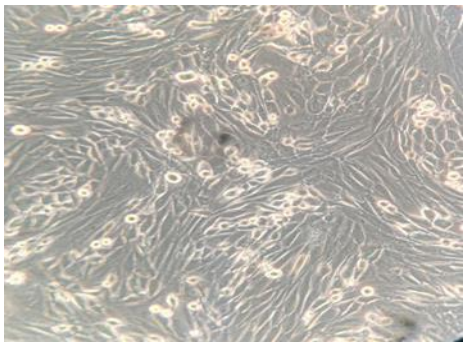

Fig.1. Rat skeletal muscle cell lines

B. Differentiation of Myoblasts into Myotubes

To differentiate myoblasts into myotubes cells were exposed to differentiation media containing 2% FBS for 7-8 days.

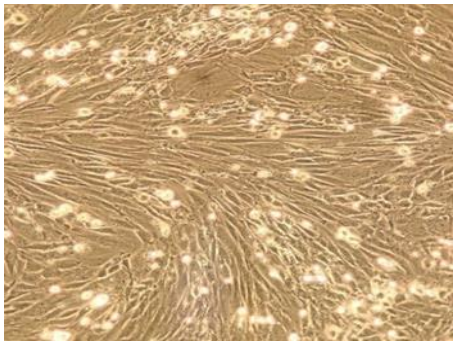

Fig.2. L6 Cells after differentiation

C. Protein extraction and estimation

Total volume of protein extracted from L6 cells exposed to 0mM and 25mM is represented in table.1.

Table.1. Total volume of protein extracted from each sample

Sample	Volume of extracted protein
Control	200 µl
0 Mm	200 µl
25 Mm	200 µl

Bradford assay is done to find out the total concentration of a protein in a sample. The concentration of a protein in a test sample is determined by comparing the result to that of a protein standard. BSA is been taken as a protein standard. Standard graph for BSA is shown in figure.3.

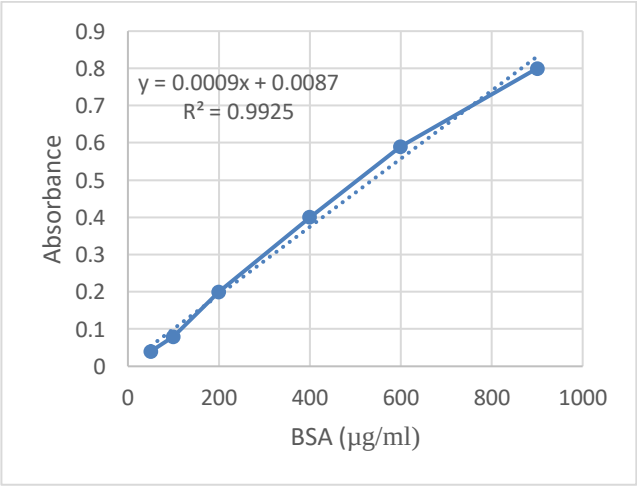

Fig.3. BSA Standard curve

The concentration of the protein was same in different samples after Bradford assay is represented in the following table

Table.2. Protein concentration of different samples

Samples	Concentration
Control	16.6 µg/µl
0Mm	17.1 µg/µl
25Mm	17.7 µg/µl

Protein concentration of cellular extracts was in the range of 16.6-17.7 µg/µl.

D. SDS-PAGE Result

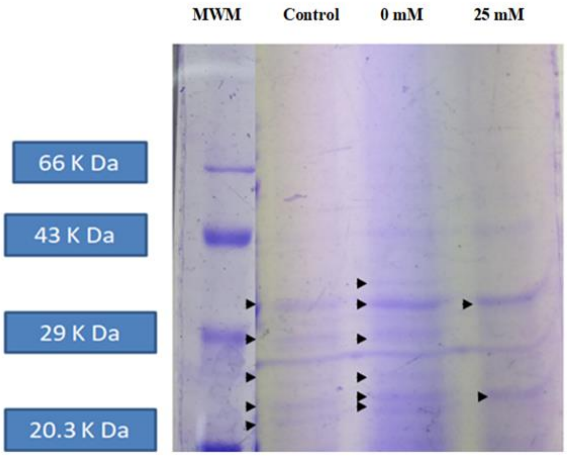

Fig.4. SDS-PAGE of total protein extract of the L6 cells exposed to different glucose concentration. (Lane 1: Medium range molecular marker, Lane 2: Control, Lane 3: 0mM, Lane 4: 25mM)

IV. Conclusion And Discussion

As proteins play relevant role in all biological functions proteomic analysis of different tissues gives useful information about the disease. Present work is aimed to study the proteomic profile of L6 cells exposed to different glucose concentrations. For this purpose L6 cells were cultured successfully. To study the effect of various glucose concentrations, L6 myoblast cells were serum starved (2% FBS) so that they can be converted into myotubes. Protein extraction and estimation was done and protein bands were observed after SDS-PAGE.

A protein band with approximately 34 KDa molecular weight appeared in all the samples loaded in the gel. This protein may be Muscle ankyrin repeat protein. This is a transcriptional regulator which helps in the expression of the protein during

recovery of the cell when they are starved. Since L6 cells were serum starved in order to initiate differentiation from myoblasts to myotubes that's why this protein appeared in all three samples (Control, 0mM, 25mM).

A protein band with approximately 28.8 KDa molecular weight appeared in Control lane and 0mM lane. This protein may be a protein called nebulin-013 which interacts to generate the mechanical force needed for muscle contraction [49]. Nebulin which plays important role in the regulation of muscle contraction is not present in 25mM lane. The absence of this protein is indicating that hyperglycemic condition is related with muscle contraction.

A protein band with approximately 25 KDa molecular weight appeared in control lane and 0mM lane. This protein may be Troponin T3 which binds with Ca(2+) and initiates the process of muscle contraction. In diabetic condition (type-2) there is change in the skeletal muscle health particularly on the growth and repair capacity [50]. Absence of this protein band in 25mM lane indicates its role in hyperglycemic condition.

A protein band with approximately 24 KDa molecular weight appeared in all the samples. This may be Caveolon-3 protein which plays a role in the sarcolemma repair mechanism of skeletal cells.

A protein band with approximately 23 KDa molecular weight appeared in Control lane and 0mM lane. This may be Synaptosomal associated protein 23 (Snap 23) which is a protein expressed in human skeletal muscle. Its role is to mediate insulin-stimulated docking and fusion of GLUT-4 cell with the plasma membrane. Since there is no insulin-stimulated docking in the diseased cell, this protein band is absent from 25mM lane.

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