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Proteomic analysis of *Substantia nigra* proteins in STZ-induced Type-II diabetic rats: A possible link with Parkinson's disease

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Type-II diabetes mellitus (TIIDM) is a metabolic disorder characterized by high level of glucose in blood due to high secretion of glucose from peripheral tissues, low secretion of insulin or dysfunction of insulin. Parkinson's disease (PD) is a neurodegenerative disorder characterized by loss of Dopamine in the *Substantia nigra* of midbrain leading to cause motor dysfunction in affected people. TIIDM and PD have associations with each other as the individuals suffering from TIIDM are at more risk to develop PD in old age. The present study has analyzed eight (08) differentially expressed proteins in the *Substantia nigra* of TIIDM rat's brain by using Nano-LC-MS/MS method. Nano-LC-MS/MS is highly recommended technique for the identification & quantification of proteins. RPS27a, PSMC1, PSMa4, ATP8, ATP5f1d and CALM3 were down-regulated while PSMa3 and PRKACa were up-regulated in rat's brain. These differentially regulated proteins were further analyzed and found to be involved in the oxidative stress, mitochondrial dysfunction, Dopamine pathway, dysfunctional insulin signaling pathway, Ubiquitin regulatory pathways, and Ca^{2+} signaling pathways of both TIIDM and PD. In conclusion, the current study proposed a link between TIIDM and PD through primary estimated involvement of these proteins in both disorders. However, more in-depth molecular and proteomics studies are needed to be carried out for the possible expression of current proteins as target in the treatment and or prevention of PD in TIIDM patients.

Keywords: Functional regulated proteins, LC-MS/MS, Oxidative stress, Proteins Interaction, Proteomics

Type-II diabetes mellitus (Non-insulin dependent DM) is a complicated metabolic disorder described by hyperglycemia due to major defects in glucose metabolism leading to production of high levels of glucose from hepatic tissues, declined secretion and defective insulin action[1,2]. Individuals affected with TIIDM are more susceptible to number of mild and severe secondary complications, therefore morbidity and mortality rates are higher in such individuals. TIIDM is very serious disease and diagnosed very late especially in developing countries with limited diagnostic facilities and minimum resources[3]. Long term hyperglycemia is linked with incurable damage and malfunction of various other body organs, mainly eyes, heart, kidneys, nerves and blood vessels leading to increased death rates and debilitations[4]. TIIDM is a long-term disease becoming one of the major public health concerns of the 21st century because it is the most prevalent disease in the world. The number of TIIDM affected people is increasing rapidly in the United States of America and remainder of the world; as 20 million individuals may have the disease in the United States alone[5,6]. The worldwide prevalence of diabetes mellitus was over 463 million in 2019, in which more than 90% of diabetes patients were diagnosed with TIIDM. The prevalence is projected to be 578 million in 2030 and 700 million in 2045[7].

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by the loss of

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dopamine-generating cells, also known as dopaminergic neurons of *Substantia nigra* in the brain leading to number of complicated symptoms and continuous loss of motor control. PD is currently the second most common neurodegenerative disease in the world after Alzheimer's disease[8]. Bradykinesia (Slowness of movement), tremor, rigidity (Muscles stiffness), and later postural instability are the classical motor symptoms of Parkinson's disease caused by deficiency of dopamine in the basal ganglia. PD is also linked with non-motor symptoms, which may become troublous symptoms in the later stages of PD[9]. Other Symptoms include dyskinesia, dementia, anosmia (absence of sense of smell), anxiety, constipation, depression, fatigue, and festination of speech, postural hypotension and micrographia[10]. Factors causing PD are unrevealed uptil now, however genetic factor and environmental factor are observed to be the two main factors that cause PD. The individual exposed more to pesticides and toxins and having head injuries are at higher risk to be affected by PD in future[11]. Although the development of PD is more common in aged individuals *i.e.*, at the age of 60 or early however it may develop earlier at the age of 20[12]. PD affects sixty thousand people in America annually and affects 7 - 10 million individuals worldwide. PD is more prevalent in men than women[13]. Parkinson's disease has affected 6·1 million people globally in 2016 as compared with 2·5 million in 1990[14].

Streptozotocin (STZ) acts as a diabetogenic agent and destroys β cells of the pancreas after gaining entry in to the cells through GLUT2 transporters when given systemically[15]. In brain GLUT2 transporters are present in certain areas including hypothalamus where these are located in neurons, endothelial cells as well as special type of ependymal cells called tanycytes[16]. GLUT2 transporters in hypothalamus possibly act as glucose sensors and control feeding behavior, thermoregulation as well as glucose homeostasis in peripheral organs[17]. Thus, it is plausible that damage to the GLUT2 containing hypothalamic cells by STZ administration led to altered effects on brain such as dysfunctional insulin signalling, oxidative stress, decreased cerebral glucose utilization, mitochondrial bioenergetics changes, behavioral impairment and neuro-inflammation in rats.

Mitochondrial dysfunction and oxidative stress contribute towards Parkinson's disease pathogenesis and potentially relevant to the link with TIIDM. Dysfunctional mitochondrial proteins create an increase in oxidative stress, neuro-degeneration and neuronal death. The features of mitochondrial dysfunction may be shared in TIIDM and PD. In Parkinson's disease, dysfunctional insulin signaling has been found to increase oxidative stress. Chronic insulin resistance in diabetes causes mitochondrial disruption and dopaminergic neuronal degeneration, resulting in dysfunctional ATP generation, fatty acid oxidation, and the generation of ROS and oxidative stress[18].

Type-II diabetes (TIIDM) and Parkinson's disease (PD) are both aging-associated diseases that are turning into epidemics worldwide. Diabetes mellitus and insulin resistance not only increase the possibility of developing Parkinson's disease but can also determine the prognosis and progression of Parkinsonian symptoms. Diabetic patients are 40% more at risk of developing PD as compared to individuals without diabetes[19,20]. TIIDM and PD Both are characterized by aberrant protein accumulation, lysosomal and mitochondrial dysfunction, dopaminergic neuronal loss (dopamine metabolism), Endoplasmic reticulum stress, oxidative stress, autophagy dysregulation, chronic systemic inflammation, deregulation of immunity and loss of central and peripheral synapse[21]. Insulin have mediative effect in regulating the glucose transport to cross the blood brain barrier (BBB) through insulin transporters to act on insulin receptors, which are widespread in all over the brain, localized on neurons, astrocytes, and microglia. Insulin resistance contributed in PD progression via increases in α-synuclein accumulation in dopaminergic neurons, oxidative stress, and mitochondrial dysfunction. Insulin resistance is a hallmark of TIIDM and may be an important contributing factor to PD too[22]. TIIDM and PD both are considered protein conformational diseases. Protein misfolding disorders (PMDs) are diseases in which misfolding, aggregation and accumulation of proteins occur in the disease-specific damaging tissues. Both TIIDM and PD are the examples of PMDs[23]. The first line of evidence that linked T2DM and PD is protein misfolding and aggregation of amyloid fibres. Amyloid fibres are synthesized from amylin [islet amyloid polypeptide (IAPP)] in TIIDM while amyloid like structures are synthesized from α-synuclein in PD[24]. To identify the molecular link between TIIDM and PD, it is crucial to identify the regulating proteins pathways in various brain regions of diabetic individuals affected with PD[25].

The current study was designed to investigate the crosstalk between TIIDM and PD by delineating the

proteins involved in molecular pathways of both diseases for designing future therapeutic strategies. Further molecular and proteomic studies would surely warranty the success of possible remedies to treat TIIDM and PD.

Materials and Methods

Institutional animal ethical statement

The animal study protocol was approved by the Ethical Review Committee of the University of Balochistan Quetta (protocol code OECD/OCDE 408), vide No. Regr/Admin/1173, dated 28 September 2012.

Development of Type II Diabetic model

Animals (*Rattus norvegicus*) administered by moderate dose of STZ and prior consumption of diet with characteristics like insulin resistance and hyperglycemia. Streptozotocin (STZ) is a highly selective cytotoxic agent used to develop a diabetic animal model causing necrosis in β-cells of pancreatic islets[26]. The experimental animal (rat) was developed as diabetic model followed the protocol of[26]. Adult male rats (*Rattus norvegicus*) of age 10 to 12 weeks, weighing approximately 190-220 grams each, have been used as animal model for induction of diabetes. The rats have reared in the cages (2 to 3 rats per cage) under ventilated room and suitable environmental conditions such as temperature 24°C (± 1°C), humidity 55% (± 5%) and 12 h light/dark cycle. The rats allowed open access to feed and water. All rats were weighted accurately and have been divided into two groups. The rats of experimental group and control group both were placed on the balanced diet. All rats were fasted for 6 to 8 h before the STZ induction. Streptozotocin (STZ) with a single dose of 65 mg/kg body weight of rat was injected intraperitoneally to experimental group using 1 mL syringes as administered with 13 mg/animal) dissolved in cold 50 mM sodium citrate buffer with pH 4.5, injected within 5 min after dissolution. Equal amount of citrate buffer (pH 4.5) was injected intra-peritoneally into the rats of control group. Permanent hyperglycemia was conformed in the rats after 4 days of injecting of STZ. Rats were provided with balanced diet and water after returning them back to the cages. The blood glucose level was measured through tail-vein blood using one Touch blood glucose meter after 4 days of administration of STZ. The rats with glucose level above 270 mg/dL have been marked as diabetic model.

Dissection of brain and extraction of *Substantia nigra*

The brains of Rats have been dissected and *Substantia nigra* has been extracted from both diabetic and control group under the protocol of[27]. A rat brain matrix, Petri dish with 5 razor blades and a #11 scalpel have been chilled on wet-ice while 2 mL microcentrifuge (labeled) tubes were placed in another container containing dry ice. A vaporizer of isoflurane was used to anesthetize the rats and waited for 3-5 min. As the rats lost consciousness, the brains of rats were removed rapidly under 2 min, rinsed with cold water and positioned in the chilled rodent brain matrix. After positioning, the brains were inserted with cold razor blades after each section of 1mm till middle way through the Pons; each subsequent blade was staggered to remove them easily. The beginning of hippocampus was noticed thoroughly when razor blades were pulling out until it was fully wrapped around midbrain. Sections have been taken through #11 scalpel razor blades. The dissection of *Substantia nigra* in rats will be expected at least in 2-3 coronal slices. The cortex and hippocampus wrapped around midbrain were removed and then made a vertical cut to separate the SN (pigmented area) from VTA (ventral tegmental area). After that we have made a horizontal cut close to the middle line over the lateral and dorsal portions of the *Substantia nigra*. At the end SN was removed from rest of the midbrain and placed the dissected tissues in the microcentrifuge tubes cooled earlier on the dry ice. Finally the tissues of SN have been stored at −80°C till further processing (Fig. 1).

Homogenization of brain tissues

The *Substantia nigra* from each brain sample was homogenized by using Overhead homogenizer D-500 (Fig. 2). 1.89% ice-cold formic acid in water with concentration of 10 mL/g tissue was used for the homogenization of tissues. Later, centrifugation was

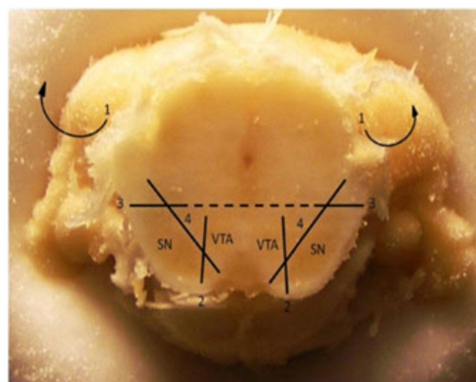


Fig. 1 — Dissection technique for the isolation of SN from brain[27]

Fig. 2 — Overhead Homogenizer D-500

performed with 14,000rpm at temperature of 4°C for 40 min. Internal standard (IS) isoprenaline was added to the supernatant at a 9:1 (*v/v*) ratio to achieve a final concentration of 500 ng/mL in order to quantify it. For protein precipitation, the IS-containing supernatant was mixed with ACN containing 1% formic acid and then, centrifugation was performed at 14,000 rpm at the temperature of 4°C for 5 min (PPT). The supernatant was extensively dried at 45°C for 1 h and 15 min in a concentrator (5301, Eppendorf, Germany). Following the PPT, the dry residue was reconstituted with the mobile phase (0.2% formic acid in water or ACN [95:5, *v/v*]) in the same volume as before the PPT. The samples were placed into vials for direct injection into the LC-MS/MS.

Sample collection and processing

After homogenization of brain tissues the brain samples have been centrifuged for 15 min at 3500 rpm and temperature was maintained at 4°C. Cellular components were removed and samples were stored at −80°C for the rest of processes. The protein concentration was measured by BCA (Bicinchoninic acid) method following the Kit protocol[28].

Protein digestion by FASP Method

FASP (Filter-aided sample preparation) method was followed for the digestion of each brain sample (200 mg) protein[29]. Following the FASP method, firstly samples have been centrifuged for 25 min at 14000 rpm setting the temperature at 20°C after concentrating the proteins in 0.5 mL KDa cutoff centrifuge filters and mixing it with 500 µL of 6 M urea in 0.1 M of tris/hydrochloric acid. The samples have been incubated for 30 min at 56°C after addition of 10 µL of 0.1 M DTT (Dithiotreitol) into the filters. After that samples have been incubated again for 30 min in darkness adding 10 µL of 0.3 M IAA (Iodoacetamide) to the filters. At the end, filters were added by 2 µg trypsin in 50 µL of 100 Mm NH4HCO3. The ratio of protein to enzyme was 50:1 and samples have been incubated for at 37°C overnight to release peptides. Finally samples have been centrifuged at 14000 rpm for 25 min at 20°C to collect the released peptides.

LC-MS/MS analysis

LC-MS/MS analysis was carried out through Nano-UHPLC coupled to MaXis II ESI-QTOF Mass spectrometer by following the protocol and parameters of[30]. The C18 reverse-phase trapping column (C18, AcclaimTM PepMapTM 100, 75 mm × 2 cm, particle size 3 mm, nanoViper, Thermo Fisher Scientificc) and C18 reverse-phase nano-LC column (75 mm × 15 cm, AcclaimTM PepMapTM RSLC C18 column, particle size 2 mm) were used to separate the protein digest, which had been dissolved in 0.1% TFA (Bruker Daltonics, Bremen, Germany). Buffer A included 0.1% formic acid in ultra-pure water, and Buffer B contained 0.1% formic acid in acetonitrile; both were used for elution of the peptide (ACN). A 2% B load was run through the system for 6 min at a flow rate of 5 mL min^{-1}, and peptides were then separated in an ACN gradient running at a flow rate of 300 mL min^{-1}. The peptides were eluted using a linear gradient from 1% to 35% B for 29 min, then up to 95% B for 2 min, held for 7 min, dropped to 2% B for 4 min, and finally equilibrated for 12 min. Positive ion mode data dependent acquisition (DDA) was performed. These standards were established: In this experiment, we used a dry gas flow rate of 4.0 L min^{-1}, a dry temperature of 180°C, an end plate offset voltage of 500 V, and a capillary voltage of 4500 V. All MS and MS/MS scans were performed between 150 and 2200 *m/z*. MS/MS was achieved by using criteria of 2500 and cycle duration of 3 sec.

LC-MS/MS data analysis using ProteinScape

Files of raw data for each run from Nano LC-MS/MS have been converted into (.xml) and then searched by applying Mascot database search engine (Version 2.6, Matrix Science, London, UK) against Swiss-Prot database. The following parameters were set to process these .xml files in the Mascot database engine. *Rattus norvegicus* in taxonomy filter, trypsin in proteolytic cleavages, missed cleavages up to 1, oxidation of methionine as variable modifications and carbamidomethyl of cysteine as fixed modifications

have been selected in each parameter. 0.08 Da for peptide mass tolerance and 0.2 Da for fragment mass tolerance have been allowed. Peptide charges were allowed at +2, +3, and +4 while accepted FDR was allowed to <1%.

Bioinformatics Analysis

Bioinformatics analysis is a fundamental and standardizedtools in order to manage all the proteins, focusing on their functional interpretation and illustrating the high degree of connectivity networks based on the existing set of proteins mining tools to screen-out their biological information from proteomics data. In addition, it provides tools for easy access to all aspects for identification, quantitation and characterization of all proteins in a cell to understand the molecular processes that mediate cellular physiology[31].

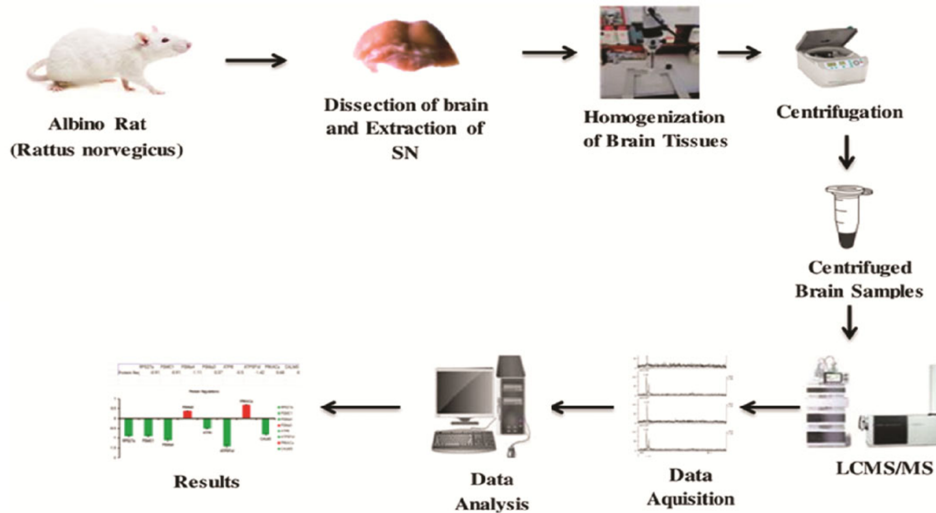
The proteins have been further implicated for bioinformatics analysis such as gene ontology (GO), Rat Genome Database (RGd), and STRING. The accession numbers of the 8 differentially regulated proteins was extracted by using the online UniProt knowledgebase database(Uniprot-kb) (https://www.uniprot.org/). Proteins were classified functionally by GOHierarchy containing three sub-Ontologies as the BP (biological process), MP (molecular process) and CC (cellular component) using PANTHER (Protein Analysis Through Evolutionary Relationships) version17.0[32].

Protein-Protein interaction among 8 differentially expressed proteins was analyzed by using STRING EMBL software (version 11.5, https://string-db.org/). Multiple proteins option was selected in search bar while *Rattus norvegicus* was opted in organisms[33].

Results

Brain dissection and isolation of *Substantia nigra* for LC-MS/MS analysis

In the present research work, Brain of diabetic and control rat were dissected to isolate *Substantia nigra* for the LC-MS/MS analysis (Fig. 3).



Schematic diagram of Research Methodology

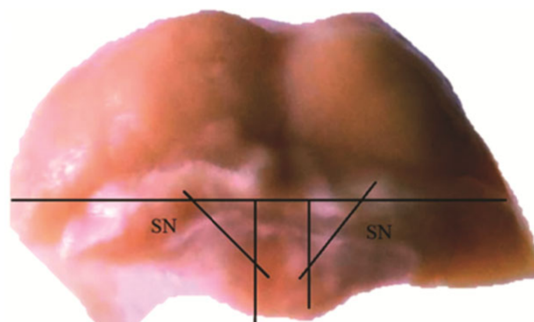


Fig. 3 — Dissection of brain and extraction of SN

After performing LC-MS/MS and bioinformatics tools, a total of 8 differentially expressed proteins were identified from both samples. Among which 6 proteins have been analyzed to be down-regulated while 2 proteins were up-regulated. RPS27a, PSMC1, PSMa4, ATP8, ATP5f1d and CALM3 were down-regulated while PSMa3 and PRKACa were up-regulated.

The results of differentially expressed proteins with accession number, gene ID, and detailed name, category of regulation, molecular weight, T-test difference and T-test p-value are presented in (Table 1). The regulations of proteins are represented in the (Fig. 4).

Functional classification of differentially regulated proteins

Functional classification of 8 differentially expressed proteins was performed by applying PANTHER GO-slim. Proteins were classified on the basis of BP, MP and CC.

Classification of differentially regulated proteins on the basis of biological processes

Differentially regulated proteins were classified on the basis of biological processes. Among these differentially expressed proteins, 4 proteins (33.3%) were involved in cellular process, followed by metabolic processes (4 proteins, 33.3%), biological regulation (1 protein, 8.3%), localization (1 protein, 8.3%), response to stimulus (1 protein, 8.3%), and signaling (1 protein, 8.3%) (Fig. 5).

Classification of differentially regulated proteins on the basis of molecular functions

Differentially expressed proteins were also classified on the basis of molecular functions, 3 proteins (50.0%) were involved in binding followed

Table 1 — Differentially expressed proteins identified in Parkinson's disease

Protein ID/Accesstion No	Gene ID RAT|RGD=	Detail Name	Category	Mol. Wt (KDa)	T-test Difference	T-test P-value
RPS27a	6489478	Ribosomal protein S27a	DOWN	17.951	-0.913942	0.043718978
PSMC1	621097	26S proteasome regulatory subunit 4	DOWN	49.184	-0.911443	0.032673827
PSMa4	61846	Proteasome subunit alpha type-4	DOWN	29.497	-1.11725	0.028715078
PSMa3	61844	Proteasome subunit alpha type-3	UP	28.419	0.379633	0.042401381
ATP8 or Mt-ATP8	621240	ATP synthase protein 8	DOWN	7.6298	-0.506901	0.030187698
ATP5f1d or ATP5d	621372	ATP synthase subunit delta, mitochondrial	DOWN	17.563	-1.42153	0.003503241
PRKACa	3389	cAMP-dependent protein kinase catalytic subunit alpha	UP	40.605	0.680129	0.003229981
CALM3	2259	Calmodulin-3	DOWN	16.837	-0.821583	0.004236917

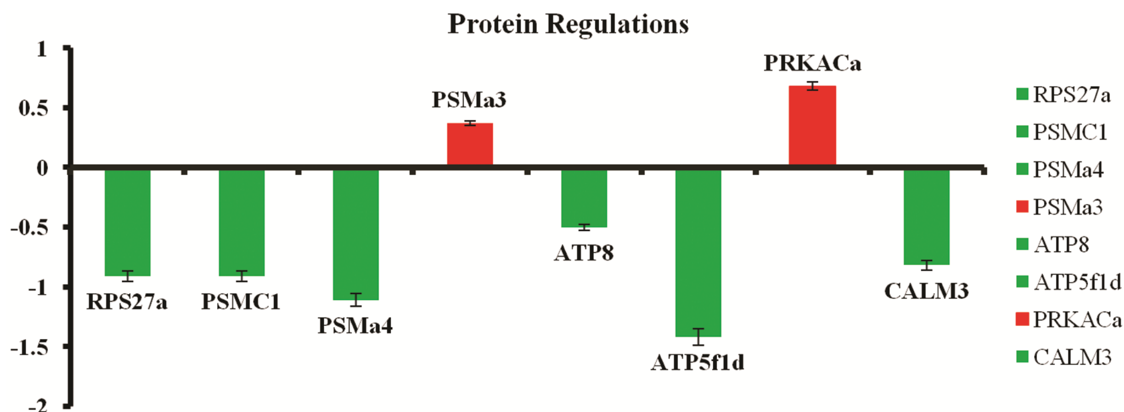


Fig. 4 — Regulation of 8 proteins presented in a bar graph

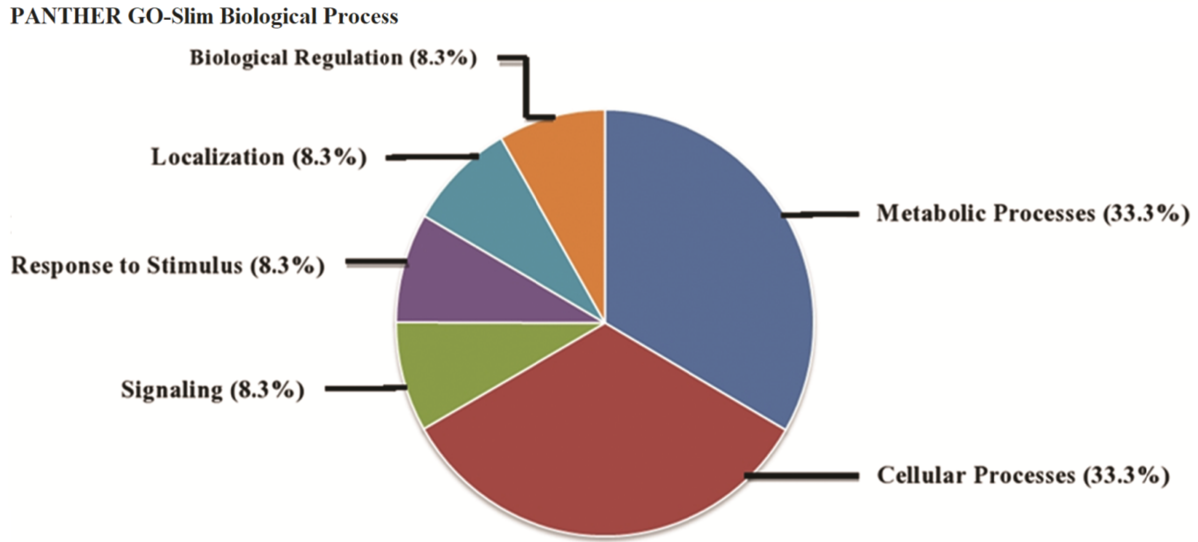


Fig. 5 — GO annotation of biological processes analyzed by PANTHER

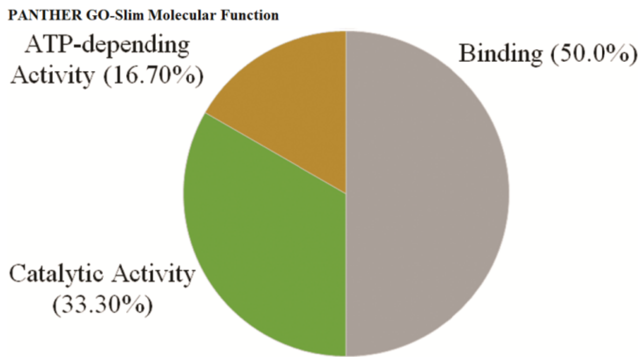


Fig. 6 — GO annotation of molecular functions analyzed by PANTHER

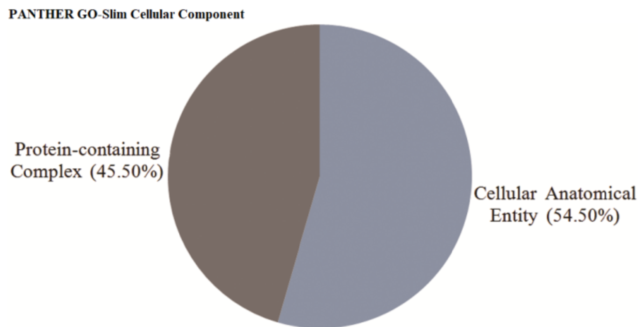


Fig. 7 — GO annotation of cellular components analyzed by PANTHER

by catalytic activity (2 proteins, 33.3%), and ATP-dependent activity (1 protein, 16.7%) (Fig. 6).

Classification of differentially regulated proteins on the basis of cellular components

Differentially regulated proteins were also classified on the basis of their cellular components

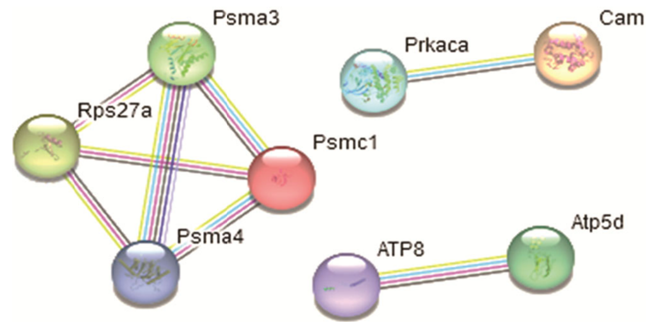


Fig. 8 — Interactive analysis of 8 differentially expressed proteins using STRING

using PANTHER. Among these proteins, 6 (54.5%) proteins were involved in cellular anatomical entity while 5 proteins (45.5%) were involved protein-containing complex (Fig. 7 & Table 2).

Interactive network analysis of differentially regulated proteins

Association and interactive network analysis of 8 differentially expressed proteins was gained by applying STRING. The interactions among these 8 differentially expressed can be analyzed as shown in (Fig 8).

The *Substantia nigra* from each brain sample was homogenized by using Overhead homogenizer D-500. 1.89% ice-cold formic acid in water with concentration of 10 mL/g tissue was used for the homogenization of tissues.

Table 2 — GO Hierarchy(three GO-Annotations) containing sub-Ontologies with differentially expressed proteins

GO Hierarchy/Annotation	GO sub-Ontologies	Differentially expressed Proteins
Biological Processes	Biological regulation (8.3%)	PRKACa
	Localization (8.3%0	ATP5f1d
	Response to stimulus (8.3%)	PRKACa
	Signaling (8.3%)	PRKACa
	Metabolic process (33.3%)	PSMC1, ATP5f1d, PRKACa, RPS27a
	Cellular process (33.3%)	PSMC1, ATP5f1d, PRKACa, RPS27a
Molecular Function	ATP-dependent activity (16.70%)	PSMC1
	Catalytic activity (33.30%)	PSMC1, PRKACa
	Binding (50.0%)	CALM3, PRKACa, RPS27a
Cellular Components	Protein-containing complex (45.50%)	PSMC1, PSMa3, PRKACa, ATP8, PSMa4
	Cellular anatomical entity (54.50%)	PSMC1, PSMa3, PRKACa, ATP8, PSMa4, RPS27a

A brief description of 08 referred proteins differentially expressed in three GO-Annotations (biological process=BP), (molecular process=MP) and (cellular component=CC) using PANTHER software

Discussion

The quantitative and qualitative analyses of proteins in brains samples of diabetic rat and control rat were performed through proteomics. Recently, NanoLC-MS/MS is mostly applied for the identification and quantification of differentially regulated proteins because it is more sensitive and rapid method as compared to other traditional methods used for proteomics.

On the basis of bioinformatics tool using the caption ‘Analysis’ of STRING tool, based on different literature reviewed and Annotated bibliography, the described eight (08) distinct proteins are biologically linked with Parkinson disease having crucial role in the pathogenesis of PD by modifying insulin signaling pathways and in many other neurodegenerative disorders as illustrated separately with particular functions.

We have found 8 differentially regulated proteins in our present research work. Among these 8 differentially expressed proteins, six proteins including RPS27a, PSMC1, PSMa4, ATP8, ATP5f1d and CALM3 have been analyzed to be down-regulated while two proteins including PSMa3 and PRKACa have been up-regulated.

The PRKACA (protein kinase Acatalytic subunit) is up regulated in the *Substantia nigra* of PD patients and involved in the pathways of dopamine and acetylcholine. It was also up regulated in various brain regions of PD mouse model. Therefore, PRKACA is involved in the progression and pathogenesis of PD through metabolic pathways[34a]. PRKACA was enriched in insulin signaling pathways therefore it could be involved in the pathogenesis of PD by modifying insulin signaling pathways[34]. On the other hand, PRKACA was up-regulated in the *Substantia nigra* of TIIDM brain samples of our study linking TIIDM with PD.

ATP5F1D was down-regulated in the spinal cord injury of rat which is involved in the pathways of number of neurodegenerative disorders, such as Parkinson disease and Alzheimer disease[35], while ATP5F1D was also down-regulated in the *Substantia nigra* of diabetic rat providing evidences for the linkage of TIIDM with PD.

The mt-ATP synthase or ATP8 is a multi-subunit complex which is very vital for the function of mitochondria and synthesis of ATP under physiological conditions. The ATP synthase enzyme is involved in various degenerative diseases of human through mutations and dysfunction of this enzyme. The activity of ATP synthase complex is affected in human diseases by altered expressions of ATP synthase genes which cause alteration in the morphology of mitochondria and cell death. PD is one of the most frequent neurodegenerative disease caused by necrosis of dopaminergic neurons in the *Substantia nigra* of brain. The pathogenicity of PD is characterized by aggregation of proteins and dysfunction of mitochondria, decreased ATP synthesis, ionic imbalance, modified Ca^{2+}-dependent transmission of impulses in the brain and cell death.

The mutations in the ATP8 gene can cause dysfunction of ATP synthase leading to pathogenesis of neurodegenerative disorders such as PD[36]. The ATP8 protein was down-regulated in the SN of TIIDM brain of present study. Therefore it is evident that TIIDM and PD have common molecular pathway for their progression.

A fusion protein consisting of ubiquitin and ribosomal protein 27a is encoded by Rps27a gene. Ubiquitin (Ub) is a protein which is responsible for many regulatory pathways in cells. Failure in the homeostasis of Ub leads to cause different type of NDDs (Neurodegenerative diseases) NDDs. Dysfunction of RPS27a plays an essential role in the development of many NDDs such as PD and AD *etc*[37]. RPS27a was down-regulated in brains of human and Rat PD model[38]. While RPS27a protein was also down-regulated in the *Substantia nigra* of diabetic type 2 brain of rat in present research work to suggest a common molecular pathway both in TIIDM and Parkinson's disease.

The UPS (Ubiquitin proteasome system) is a complex pathway which is involved in cellular physiology and breakdown of Toxic and damaged proteins. The dysfunction of UPS is proposed to play an essential role in the development of PD because an UPS impaired mouse model has been characterized by degeneration of dopaminergic neurons and aggregation of syncline. 26S proteasome is the most familiar type of UPS. PSMC1 (26S proteasome regulatory subunit 4, ATPase 1) is an ATPase enzyme which is encoded by PSMC1 gene. The *Substantia nigra* of a PSMC1 conditional knockout mice have been characterized by depletion of 26S proteasome causing neuro-degeneration like PD. It has been hypothesized that variation in PSMC1 may alter the function of UPS leading to cause PD through accumulation of proteins[39]. PSMC1 has also been to be down-regulated in our research study which reveals that UPS especially PSMC1 plays an important role in the development of both TIIDM and PD.

PSMA4 (proteasome subunit alpha type-4) is another important protein which is a member of the (UPP) ubiquitin-proteasomal pathway playing a crucial role in PD[40]. PSMA4 was down-regulated in the in the TIIDM brain sample of our study however it was overexpressed in the blood samples of PD patients which predict that it is significantly involved in the pathogenesis of PD. Multiple cohort and bioinformatics studies suggested that these findings can predict the causes and molecular pathways of PD which is helpful in the therapy of PD[41].

CaM (Calmodulin) or CALM is a Ca^{2+}-binding protein which is highly expressed in the brain. CaM is upregulated when concentration of Ca^{2+} is high inside the cells leading to apoptosis. CaM regulates the CaMBPs (calmodulin binding proteins) which play an important role in the brain by regulating the activity of synaptic proteins and plasticity of neurons. Dysregulation of CaMBPs has been associated with the pathogenesis of many neurodegenerative diseases such as PD. Increased level of [Ca^{2+}] has been analyzed in the neurons of various regions of PD brain leading to cause death of neurons. CaM is interacted with CaMBPs in high level [Ca^{2+}]i which are associated with homeostasis of Ca^{2+},Ca^{2+}-signaling pathways and other pathways in the neurons. CaM is highly expressed to activate more CaMBPs in PD[42]. CALM3 is a subunit of CALM which was down-regulated in the *Substantia nigra* of brain of TIIDM rat model. PSMA3 was up-regulated in the SN of TIIDM rat model[43].

Conclusion

In conclusion of our research study, 8 proteins have been revealed to be differentially regulated in the *Substantia nigra* of the brain of diabetic rats model. These proteins were involved in oxidative stress, mitochondrial dysfunction, Endoplasmic reticulum stress, dopaminergic neuronal loss, autophagy dysregulation, chronic systemic inflammation, deregulation of immunity, loss of central and peripheral synapse, DA pathways, Ubiquitin regulatory pathways, UPS pathways, and Ca^{2+} signaling pathways. Dysregulation of these differentially expressed proteins were found to be involved in the progression of TIIDM and PD both. Although the findings of the present study propound that TIIDM and PD are interlinked through different molecular pathways but it is too early to conclude that TIIDM is involved in the pathogenesis of PD. On the basis of above investigated proteins, further proteomics research studies are recommended to reveal the linkages between TIIDM and PD.

Conflict of interest

All authors declare no conflict of interest.

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