Expression Study Of TMEM229B Genes In Relation With Apoptotic Pathway Of STZ Induced Diabetic Rats

Muhammad Aleem[1], Yasir Nawaz[2]*, Saba Munir[2], Aqeela Nawaz[2], Ubaid Ullah[3], Sara Parveen[4], Maria Hussain[2], Alia Iqbal[2], Muhammad Hasan Ilyas[3], Allah Ditta, Muhammad Ashfaq, Fouzia Tanvir[2]

[1]Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan
[2]*Department of Zoology, Faculty of Life Sciences, University of Okara, Okara, Pakistan
[3]Department of Zoology, Government College University Lahore, Pakistan
[4]Department of Zoology, University Of Education Lahore, Pakistan

***Corresponding author:** Yasir Nawaz
*Email: royyasirnawaz@gmail.com

Abstract

Background: Diabetes mellitus (DM) is a chronic non-communicable endocrine disorder characterized by hyperglycemia. Streptozotocin (STZ) is a naturally occurring compound which damages pancreatic β cells and has been broadly implemented to produce an animal model of DM. The cytotoxic effect of STZ accompanying the synthesis of ROS bases oxidative stress and oxidative injury in the cells. **Objectives:** In the present study, the effect of STZ induction on the apoptotic genes as well as the glucose metabolism has been investigated. **Methods:** The two groups of albino rats (each n=6) were taken. One group has been considered as the control and the other was STZ induced diabetics. **Results:** It has been shown that the blood sugar levels of STZ treated rats showed a significant increase as compared to the control group, whereas significant decrease ($p < 0.05$) in the average body weights over a period of two weeks has been observed. This gradual and significant increase in blood sugar and decrease in weights of experimental group disclosed that STZ effectively had induced diabetes in treated rats. The histopathological results of diabetic pancreas has shown lower concentration of beta cells in Langerhans islets, Acinar cell necrosis and inflammatory cell aggregation around beta cell while they are normal in control group without any inflammation. **Conclusion:** To conclude, the expression of apoptotic genes like BAX, p53 and Caspase 3 has been upregulated markedly in STZ induced group whereas the level was normal in the control group. All the data was normalized using GAPDH as an internal control which remained consistent in both groups. Furthermore, the expression of TMEM229B which is a major contributor of glucose metabolism has also been studied and it was intensely repressed by STZ.

Keywords: Diabetes mellitus; STZ; STZ-Induced diabetes; ROS; TMEM229B; TIIDM

Introduction

Diabetes mellitus (DM) is a chronic non-communicable endocrine disorder characterized by hyperglycemia, which can be caused by a deficiency of insulin secretion by the pancreatic β-cells or by insensitivity of target tissues to insulin [1]. Diabetes Mellitus (DM) is one of the most widespread diseases globally with a growing rate. It is a huge challenge to public health as a principal reason of morbidity and mortality. It has reached epidemic proportions worldwide, and has appeared as a great socioeconomic problem for the developing realm [2]. Pakistan is one of the great prevalence areas, presently having 6.9 million affected individuals, with estimates likely to double by 2025. Pakistan presently standing at 7th position among the countries with main problem of DM and it is anticipated to move to 4th position if current condition continues [3].

Streptozotocin (STZ) is a mixture of α- and β-stereoisomers which occurs as light yellow or white crystal-like powder. showing broad-spectrum antibiotic and antineoplastic characteristics, isolated from a soil bacterium Streptomyces achromogenes. Amongst the diabetic chemicals, STZ is the most ideal drug to yield an animal model of human DM because STZ-induced diabetes functionally, structurally and biochemically looks like human DM [4,5].

Streptozotocin has been broadly implemented to produce an animal model of T1DM and T2DM either individually, or by combining with high fat diet or nicotinamide administration. DM is linked to oxidative stress, and causes increased synthesis of ROS [6,7]. The cytotoxic act of STZ accompanying the synthesis of ROS bases oxidative stress and oxidative injury in the cells [8]. Extra nitric oxide and oxygen free radicals in reaction to STZ action causes death of cell at early phases. Furthermore, STZ damages mitochondrial respiratory complex, hinders aconitase activity, and alters mitochondrial membrane potential, causing the disturbance in mitochondrial bioenergetics [9]. STZ in the mitochondria speed up oxidative stress in pancreatic β-cells [10]. p53-responsive genes are up regulated in islets of STZ treated sample [11]. STZ treated rats shows increase in Bax protein expression when compared with non-diabetic control group. Caspase-3 activity level also found higher in diabetic group as compared to control group [12]. A transmembrane protein (TMEM229B) is a kind of proteins which extends through biological membranes. Many of which spans the lipid

bilayer of the cell membrane but various are located at the membrane of organelles. The TMEM229B family contains proteins of generally indefinite occupations. Several studies presented that TMEM229B expression can be decreased or enhanced in tumor cells as compared to that of neighboring normal cells. [13]. Present study was aimed at the evaluation of STZ- induced diabetes and the expression of TMEM229B gene in the pancreatic beta cells of mice by apoptotic pathways.

Materials and methods

Sample Collection and Study Design

Twelve Male albino rats were brought to animal house and selected rats were retained in polypropylene under controlled conditions maintaining a temperature of 20-25°C and providing a 12 hr light/dark cycle, regular rodent pellet diet and water to rats. The procedure for rat study was carried out after approval by Animal Ethical Committee of The University of Lahore and all monitoring was done according to the guidelines provided by the committee. Study was performed in university Lab, institute of molecular biology and biotechnology. The 12 healthy rats were selected for the study and then were randomly categorized into 2 groups; (n=6). Groups were designated as follows: Group I: (Control) receiving no treatment. Group II: (Experimental) treated only with STZ.

Induction of STZ

Prior to STZ induction it was ensured that the rats were fully fed and grown and were able to tolerate STZ induced diabetes as well. Streptozocin was injected intraperitoneally into each of the rat of group 2 i.e. experimental group. The rats of group 1 i.e. control group received no STZ. Dose of STZ given to each rat was according to its body weight. Amount of dose induced was calculated by using the formula:

$$\text{Dose} = \frac{55}{1000} \times \text{Body weight of rat}$$

Thus, calculated amount of STZ dose were injected into each rat. 1 cc syringes were used to inject STZ in mice intraperitoneally with great care.

Induction of Nicotinamide

30 minutes after the administration of streptozotocin (STZ), nicotinamide was induced in mice. Amount of dose of nicotinamide was calculated by the following formula.

$$\text{Dose} = \frac{120}{1000} \times \text{Body weight of rat}$$

Thus, calculated amount of NA dose was then injected to the mice.

Measurement of Blood Glucose

After administration of both STZ and nicotinamide glucose level in plasma was observed regularly using glucometer for confirmation of diabetes induction.

Dissection

At 15th day after the administration of nicotinamide, the mice were dissected pancreas were taken out and each divided into two halves, one half was stored in 1ml trizole for later use, while the other one was stored in refrigerator with 1ml formalin. Formalin was used to preserve the pancreas in stable form to avoid any disruption and contamination, while the trizole was used to maintain the RNA integrity during homogenization as well as to assist in disrupting other cells leaving only RNA in the aqueous phase.

RNA Extraction and PCR

RNA was extracted from pancreatic tissues of both groups (Control and experimental) by employing standard Trizol method according to given protocol (Shabbir, 2004). cDNA was synthesized using RNA sample with M-MLV reverse transcriptase according to manufacturer's protocol (Invitrogen, Inc. USA). GAPDH was used as positive control. For investigation of gene expression before and after treatments PCR was performed following the standard protocol provided by the manufacturer (Sigma-Aldrich). Then the gel electrophoresis was performed for visualizing the final products of PCR according to the manufacturer's guide (Addgene).

Histological examination

The pancreatic tissues were fixed in 10% formalin and subjected to HE staining, then examined under high power microscope.

Statistical analysis

All of the data was systematically organized and then entered on the data sheet using Microsoft Excel 2016. The data was analyzed by applying t-test, using SPSS (Statistical Package for Social Sciences version 25). Data regarding gene expression was analyzed using Graph Pad Prism 5 software and relevant graphs were obtained.

Results

Blood Glucose Level

Blood glucose levels were measured for each of the mice from both groups at regular intervals for the confirmation of diabetes induction by STZ (Figure 1) (Table 1). A gradual increase in blood glucose level of experimental group was observed starting after STZ induction till the dissection process. Independent sample t test was performed to assess any difference between the average glucose level of experimental and control groups. A significant difference ($p < 0.05$) between the mean glucose values of two groups was observed over a period of three weeks (Table 3).

Body Weights

Animals were weighed after equal interval of time starting from day 1 until the end of experiment (Figure 1) (Table 2). A significant difference ($p < 0.05$) between the average body weights of two groups was observed over a period of two weeks (Table 3).

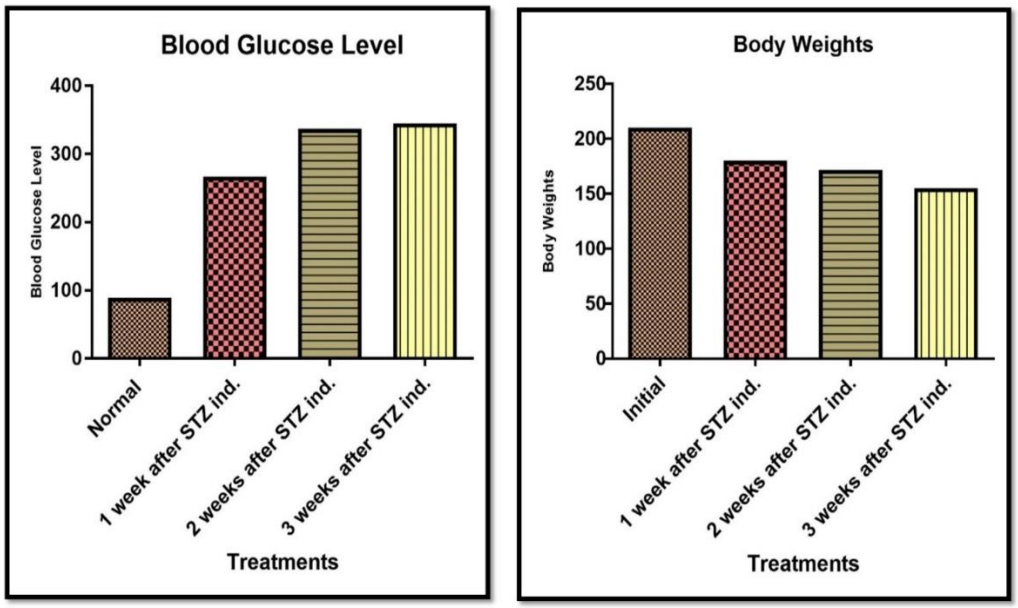

Figure 1: indicates the blood glucose level and body weight of rats

Histopathological Analysis of pancreatic tissues:

Control group results

Histological examination of the pancreatic tissue revealed normal looking endo-and exocrine elements of pancreas. The Islets of Langerhans was containing normal looking Beta cells concentration. The acinar cells also appeared normal. No evidence of any degeneration, inflammation, calcification, granuloma, or malignancy was seen (Figure 2a).

Experimental group results

Histological examination of the pancreas revealed marked fatty infiltration and shrinking of islets cell. The Islets of Langerhans contained below to normal Beta cells concentration. The inflammatory cells aggregation was also present around Beta cells. Acinar cell necrosis was also evident. No atypia or malignancy was seen (Figure 2b).

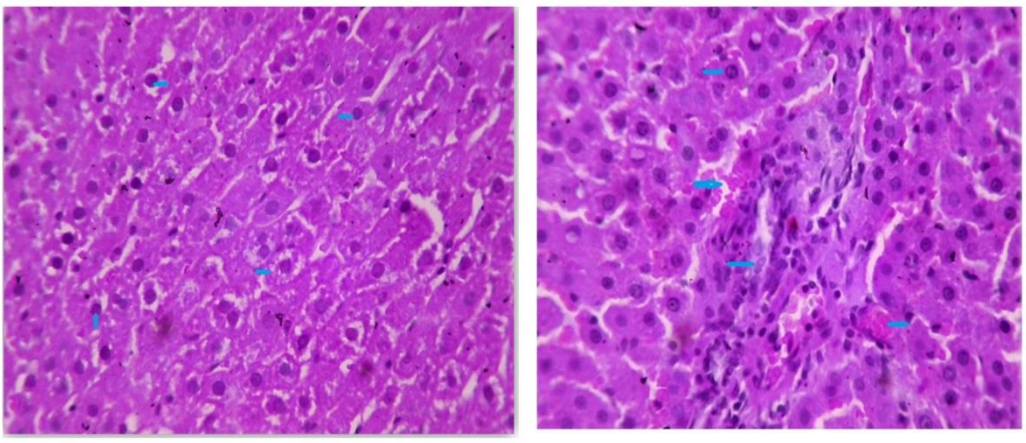

Figure 2a Figure 2b

Figure 2: Indicates the histology of pancreatic tissues in control and experimental groups

GAPDH

GAPDH is used as an internal reaction control for qPCR, because the GAPDH gene is expressed at higher levels and is regarded as a reference gene. Its expression level is not influenced by experimental elements. Current results show that that GAPDH is fully expressed and any variation occurring in the target genes would be due to the effect of experimental factors and not due to the flaws of the technology used and preparative techniques (Figure 3).

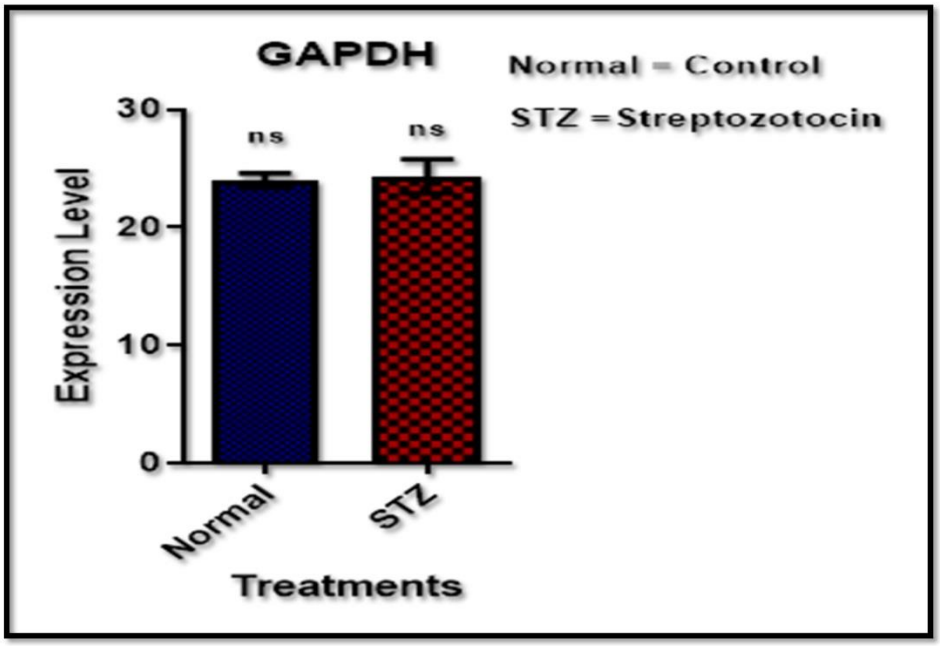

Figure 3: Level of GAPDH Expression in Control and Experimental groups

Gene expression in pancreatic beta cells

Our findings reveals that the expression of apoptotic genes like p53, BAX and Caspase 3 has been upregulated markedly in STZ induced group whereas the level was normal in the non-diabetic group (Figure 4). Furthermore, the expression of TMEM229B which is highly expressive in untreated group has also been studied and found intensely suppressed by STZ (Figure 5).

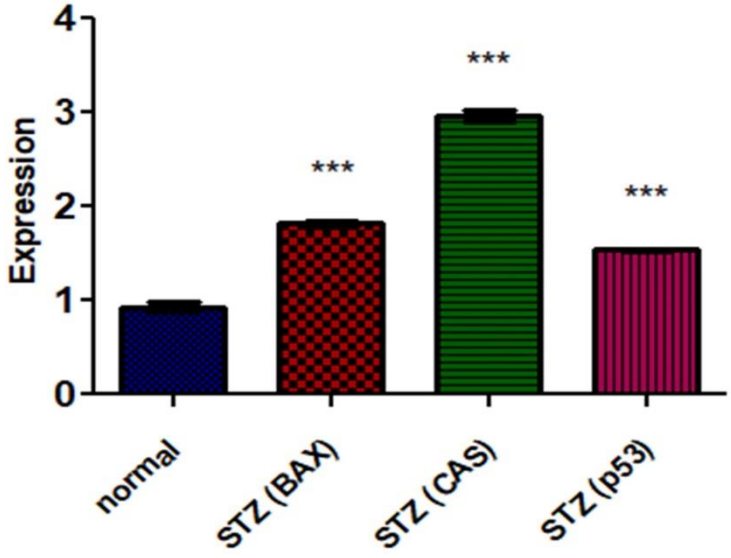

Figure 4. The expression of BAX, Caspase 3 and p53 has shown upregulated expression in STZ induced group and control group

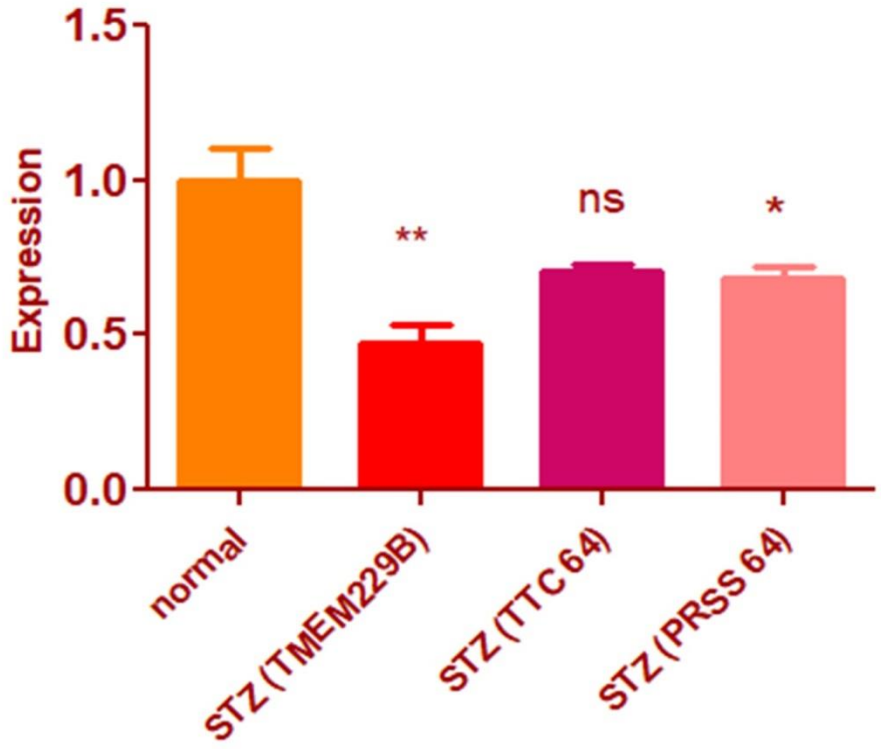

Figure 5: Genes involved in glucose metabolism

The tables in the text show details as follows:

Discussion

Table 1: Blood sugar level of control and experimental group mice in mg/dL (mean ± SEM)

Parameter	Group	Mean ± SD	t-value	p-value
Blood Glucose Level	Experimental	316.3 ± 22.2	15.83	0.00*
	Control	95.1 ± 9.4		
Body Weight	Experimental	182 ± 2.2	-6.78	0.002*
	Control	231 ± 12.3		

Table 2: Body Weights of control and experimental group mice in gm (mean ± SD)

Group	Body Weights of rats in gm (mean ± SD)			
	Initial	Day 1	Day 7	Day 14
Group – I (n=6) Control (untreated)	209.0 ± 21.23	217.5 ± 16.2	234.5 ± 4.9	241.5 ± 0.70
Group – II (n=6) Experimental (Treated with STZ)	196.5 ± 57.2	184.5 ± 58.6	181.5 ± 3.5	180.0 ± 4.2

Table 3: Comparison of Means Between Two Groups using Student's t-test

Parameter	Group	Mean ± SD	t-value	p-value
Blood Glucose Level	Experimental	316.3 ± 22.2	15.83	0.00*
	Control	95.1 ± 9.4		
Body Weight	Experimental	182 ± 2.2	-6.78	0.002*
	Control	231 ± 12.3		

Diabetes mellitus (DM) is a hyperglycemic chronic condition that causes insulin insufficiency due to destruction of beta cells. It corresponds to a group of metabolic disorders retaining high blood glucose levels. Deficient action of insulin is caused by insufficient secretion of insulin or reduced responses of tissue to insulin [14]. In DM, pancreatic β-cells in the islets of Langerhans are damaged gradually, reducing endogenous production of insulin leading to a low insulin level. This results in insulin hyposensitivity, causing hyperglycemia [15].

Prolonged contact to hyperglycemia is a disproportion in glucose breakdown, upsurges the production of free radicals [16]. Free radicals are accountable for the pathogenesis of diabetes and the incidence of diabetic problems. The augmented levels of blood sugar in diabetes are linked with high lipid peroxidation (LPO), that may result in, chronic

tissue destruction [17]. Streptozotocin (STZ), an extensively used chemical to cause investigational diabetes in organisms, can prompt pancreatic beta cell destruction through enhancing reactive oxygen species formation [18]. Several studies have established that STZ causes reproductive noxiousness chiefly through forming ROS and nitric oxide in vitro and in vivo [19]. It is confirmed that STZ can cause cell noxiousness in human hepatic cells by enhancing ROS formation, oxidative stress and mitochondrial malfunction [20].

Glucose is mainly responsible for the secretion of insulin and the basic cause of insulin secretion pathophysiology. If hyperglycemic conditions persist for long can cause a glucose toxicity of beta cells ultimately causing apoptotic beta cell destruction, it is stated that TIIDM is associated with apoptotic destruction of beta pancreatic cells [21]. One of the studies revealed that pancreatic cells of TIIDM patients were shown to exhibit apoptosis just after mitosis. TIIDM and TIIDM have a common feature regarding their pathophysiology i.e. apoptotic cell death of beta cells [22]. In case of both the TIDM as well as TIIDM the beta cells of pancreas are shown to undergo destruction or a loss of beta cells is observed this is due the increased apoptosis in the cells [23].

In current studies streptozotocin treated mice showed a significant increase in the levels of blood sugar as compared to the mice of control (untreated) group, which showed that STZ effectively had induced diabetes in experimental group. However, the mice of control group were having glucose level within normal range. In numerous low dose STZ controlled mice a substantial increase in blood glucose was detected. A continuing and progressive rise in Streptozotocin-induced diabetes in the mice and blood glucose level was detected and animals develop type I or insulin dependent diabetes. The results of current study are consistent with an earlier study. Studies of the STZ induced diabetes exposed that intraperitoneally injected many low doses of STZ (40 mg/kg) caused diabetes in mice (with blood sugar level < 250 mg/dl) after 2 to 3 weeks of STZ injection. Several low dose STZ diabetes in mice is believed to look like human type I diabetes in numerous features. Nevertheless, there is a conflict regarding this issue and various authors revealed that administration of manifold low dose STZ injection in the few preliminary days' cause Type 2 diabetes and produce Type 1 diabetes in progression [24].

Present study indicated significant decrease ($p < 0.05$) in the average body weights of STZ treated group over a period of two weeks. This gradual and significant decrease in weights of experimental group enabled us to claim that STZ had effectively induced the diabetes in mice. These results were in agreement with many others. In a study employing two practices for developing diabetes, multiple low doses of STZ (MLDS) and one high dose of STZ (OHDS), the preliminary weights of the mice were same in control and diabetic groups. Three days after the STZ administration, a substantial decrease ($p <0.05$) in body weight of OHDS groups (130 and 150 mg/kg) as compare to that of control group as a distinctive aspect for diabetic status was observed. This body weight decrease continued during the study. Multiple low doses of STZ triggered no decrease in body weight [25].

In our study, the destruction of pancreas in STZ induced diabetic mice was observed. Current studies showed results similar to that of many other researchers regarding histopathological examination of the pancreas presenting normal islets with clusters of purple stained beta cells for control mice. However, in the STZ-induced diabetic mice, pancreas showed destruction or absence of islets cells as compare to that of control mice [26]. Streptozotocin can induce breakdown of the pancreas with a lobular disintegration and a decline in degree and amount of cells in islets of Langerhans. [27,28].

Regardless of its widespread use in diabetes investigation, yet, the effect of STZ administration on pancreatic beta cells, particularly on the global gene expression profile in vivo, stays vague. In contrast to many others our study revealed that STZ significantly suppress the gene TMEM229B which is major contributor in glucose metabolism. TMEM229B is a transmembrane proteins which found in biological membranes like lipid bilayer of the cell membrane and at the membrane of organelles [14]. One of the studies showed that the injection of STZ stimuluses p53-responsive genes in affected cells, together with p21, the gene encoding for a cell cycle capture mediator. STZ administration is also revealed to overwhelm the expression of a group of genes associated with key beta cell activities or development of diabetes, proposing the occurrence of global beta cell imperfections in STZ affected islets. The group recognized diabetes linked genes which are extremely expressed in control group islets and intensely inhibited by STZ, including Tmem229B [7].

Conclusion

In conclusion, Streptozotocin treated mice shows significant increase in the levels of blood sugar as compared to the mice of control (untreated) group, which shows that STZ effectively had induced diabetes in experimental group. A gradual and significant decrease in weights of STZ treated group further strengthen our results elaborating that STZ lead to induce the diabetes in mice. Induction of STZ causes panctreatic beta cell destruction by activating the cellular apoptotic pathway leading to Induction of diabetes mellitus type 2 in mice. Intraperitoneal streptozotocin administration leads to the upregulated expression of apoptotic genes and suppression of TMEM229B gene in the pancreatic beta cells of mice.

Acknowledgments

The authors sincerely thank to department in University for the technical assistance on the use of the animal facility, helping in animal feeding and dissection and for carrying out few biochemical assays.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Surya S, Salam A D, Tomy D V, Carla B, Kuma R A and Sunil C. Diabetes mellitus and medicinal plants-a review. Asian Pacific Journal of Tropical Disease.2014;4(5):337-347.
2. Ashraf S, Iftikhar and Qayum S B. Utilization of Newly Established Executive Health Services at Rehman Medical Institute, Peshawar, Khyber Pakhtunkhwa, Pakistan.Journal of Rehman Medical Institute. 2017;3(1-2):19-25.
3. Qidwai W and Ashfaq T. Imminent epidemic of diabetes mellitus in Pakistan.issues and challenges for health care providers. 2010;9 (3):112
4. Eleazu CO, KC Eleazu, S Chukwuma and Essien U N. Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals; its practical use and potential risk to humans. Journal of Diabetes & Metabolic Disorders.2013;12 (1):60.
5. Omolaoye T S, Skosana B T and du Plessis S S. Diabetes mellitus-induction; Effect of different streptozotocin doses on male reproductive parameters. Acta histochemical.2018;120(2):103-109.
6. Tang C L, Zou J N, Zhang R H, Liu Z M and Mao C L. Helminths protect against type 1 diabetes; effects and mechanisms. Parasitology research.2019;118(4):1087-1094.
7. Szkudelski T. Streptozotocin–nicotinamide-induced diabetes in the rat; Characteristics of the experimental model. Experimental biology and medicine.2012;237(5):481-490.
8. Lenzen S. The mechanisms of alloxan-and streptozotocin-induced diabetes, Diabetologia.2008;51(1):216-226.
9. Al Nahdi A M, John A and Raza H. Elucidation of molecular mechanisms of streptozotocin-induced oxidative stress, apoptosis, and mitochondrial dysfunction in Rin-5F pancreatic β-cells. Oxidative medicine and cellular longevity. 2017;2017(1):1-15.
10. Palsamy P and Subramanian S. Ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia mediated oxidative stress, and pancreatic β-cell dysfunction in streptozotocin-nicotinamide-induced diabetic rats. Journal of Cellular Physiology.2010;224(2):423-432.
11. Tonne, J. M., Sakuma, T., Deeds, M. C., Munoz-Gomez, M., Barry, M. A., Kudva, Y. C., & Ikeda, Y. (2013). Global gene expression profiling of pancreatic islets in mice during streptozotocin-induced β-cell damage and pancreatic Glp-1 gene therapy. *Disease models & mechanisms*, *6*(5), 1236-1245.
12. Anarkooli, I. J., Sankian, M., Vahedi, F., Bonakdaran, S., Varasteh, A. R., & Haghir, H. (2009). Evaluation of insulin and ascorbic acid effects on expression of Bcl-2 family proteins and caspase-3 activity in hippocampus of STZ-induced diabetic rats. *Cellular and molecular neurobiology*, *29*(1), 133-140.
13. Schmit K and Michiels C. TMEM proteins in cancer: a review. Frontiers in pharmacology.2018;9(1):1345.
14. Czech M P. Insulin action and resistance in obesity and type 2 diabetes. Nature medicine.2017;23(7):804-814.
15. Raish M, Ahmad A, Jan B L, Alkharfy K M, Ansari M A, Mohsin K and Al-Mohizea A. Momordica charantia polysaccharides mitigate the progression of STZ induced diabetic nephropathy in rats. International journal of biological macromolecules. 2016;91(1):394-399.
16. Saravanan G and Ponmurugan P. Ameliorative potential of S-allyl cysteine on oxidative stress in STZ induced diabetic rats. Chemico-biological interactions.2011; 189(1-2):100-106
17. Bhor V M, Raghuram N and Sivakami S. Oxidative damage and altered antioxidant enzyme activities in the small intestine of streptozotocin-induced diabetic rats. The International Journal of Biochemistry & Cell Biology.2004;36(1):89-97.
18. Chen F, Xiong H, Wang J, Ding X, Shu G and Mei Z. Antidiabetic effect of total flavonoids from Sanguis draxonis in type 2 diabetic rats. Journal of ethnopharmacology.2013;149(3):729-736.
19. Seo E, Lee E K, Lee S, Chun K H, Lee M Y and Jun H S. Psoralea corylifolia L. seed extract ameliorates streptozotocin-induced diabetes in mice by inhibition of oxidative stress. Oxidative medicine and cellular longevity.2014;2014(1):1-9.
20. H Raza and A John. Streptozotocin-induced cytotoxicity, oxidative stress and mitochondrial dysfunction in human hepatoma HepG2 cells. International Journal of Molecular Sciences.2012;13(2012):5751-5767.
21. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. British journal of cancer. 1972;26(4):239-57.
22. Mandrup-Poulsen T. beta-cell apoptosis: stimuli and signaling. Diabetes. 2001 1;50(suppl 1): S58.
23. Lee SC, Pervaiz S. Apoptosis in the pathophysiology of diabetes mellitus. The international journal of biochemistry & cell biology. 2007 1;39(3):497-504.
24. Arora S, Ojha S K and Vohora D. Characterisation of streptozotocin induced diabetes mellitus in swiss albino mice. Global Journal of Pharmacology.2009;3(2):81-84.
25. Ventura-Sobrevilla J, Boone-Villa V D, Aguilar C N, Román-Ramos R, Vega-Avila E, Campos-Sepúlveda E and Alarcón-Aguilar F. Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice. In Proc West Pharmacol Soc.2011;54(1):5-9.
26. Sellamuthu P S, Arulselvan P, Muniappan B P, Fakurazi S and Kandasamy M. Mangiferin from Salacia chinensis prevents oxidative stress and protects pancreatic β-cells in streptozotocin-induced diabetic rats. Journal of medicinal food.2013;16(8):719-727.

27. Pari L and Sankaranarayanan C. Beneficial effects of thymoquinone on hepatic key enzymes in streptozotocin–nicotinamide induced diabetic rats. Life sciences.2009;85(23-26):830-834.
28. Alimohammadi S, Hobbenaghi R, Javanbakht J, Kheradmand D, Mortezaee R, Tavakoli M and Akbari H. RETRACTED ARTICLE: Protective and antidiabetic effects of extract from Nigella sativa on blood glucose concentrations against streptozotocin (STZ)-induced diabetic in rats; an experimental study with histopathological evaluation. Diagnostic pathology.2013;8(1):137.