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Swertisin, a novel SGLT2 inhibitor, with improved glucose homeostasis for effective diabetes therapy

Gurprit Bhardwaj [a], Mitul Vakani [a], Abhay Srivastava [b], Dhaval Patel [c], Anju Pappachan [c,d], Prashant Murumkar [e], Hemal Shah [a], Rushabh Shah [a], Sarita Gupta [a,*]

[a] Molecular Endocrinology and Stem Cell Research Laboratory, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, 390021, India
[b] Regenerative Medicine Program, Institute of Cardiovascular Sciences, Department of Physiology and Pathophysiology, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, MB, Canada
[c] Department of Bioinformatics and Structural Biology, Indian Institute of Advanced Research, Koba, Gandhinagar, 382007, Gujarat, India
[d] School of Life Sciences, Central University of Gujarat, Gandhinagar, 382030, Gujarat, India
[e] Faculty of Pharmacy, Kalabhavan Campus, The Maharaja Sayajirao University of Baroda, Vadodara, 390001, Gujarat, India

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ABSTRACT

Failing pancreas and subsequent loss of pancreatic β cells worsen diabetic conditions which are further alleviated by the mounting up of glucose levels. Inhibition of sodium glucose cotransporter 2 (SGLT2) in the kidney responsible for glucose reabsorption strikingly reduces blood glucose levels. Bioactive swertisin showed a promising glucose-lowering effect. Hence, we aimed to mechanistically dissect the glucose lowering property of swertisin. A systematic *in silico, in vitro,* and *in vivo* approach was directed for target analysis of swertisin. Molecular docking was performed with Swertisn-hSGLT2 complex. Glucose uptake assay and protein expression for SGLT2 and regulatory proteins were performed under swertisin effect. Various physiological and metabolic parameters were evaluated in STZ induced BALB/c mice using swertisin treatment. SGLT2 expression was evaluated in the kidney tissue of mice. Swertisn-hSGLT2 molecularly docked complex showed similar binding energy compared to the Canagliflozin-hSGLT2 complex. Swertisin inhibited glucose uptake and decreased expression of SGLT2 in HEK293 cells. Swertisin does not affect GLUT mediated glucose transport. Swertisin treated diabetic mice demonstrated remarkable improvement in overall glucose homeostasis. Reduced expression of SGLT2 was found in kidney tissue along with reduced PKC expression which is one of the key regulators of SGLT2. Our study explored SGLT2 as a selective target of swertisin for its swift glucose-lowering action which not only inhibits SGLT2 but also reduces its expression in diabetic condition. Thus, the potential property of swertisin as a glucose-lowering agent is remarkable which points towards the likelihood of a wider avenue of diabetes therapy.

1. Introduction

A state of hyperglycemia is a hallmark characteristic of diabetes mellitus. Sustaining good glycaemic control is a pre-requisite of any effective diabetes therapy. It has been demonstrated that the kidney apart from other organs plays a key role in maintaining normoglycemia in blood.97% of reabsorption of glucose occurs via Sodium glucose co-transporter 2 (SGLT2) and 3% by SGLT1. But this mechanism becomes malabsorptive in diabetes [1] and hence hyperglycemia persists. Hyperglycemia also affects certain protein kinases that are involved in the regulation of SGLT [2,3].

SGLT2 inhibitors are highly explored current class of drugs not only in type 2 diabetes but also in type 1 diabetes as its mechanism is independent of circulating insulin or insulin sensitivity [1]. Gliflozins are

Abbreviations: SGLT2, Sodium glucose co-transporter 2; SGLT1, Sodium glucose co-transporter 1; EL, *Enicostemma Littorale*; HEK293, Human embryonic kidney 293 cell line; Caco2, Cancer coli-2 (human colorectal adenocarcinoma cell line); 2-NBDG, (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose); GLUT, Glucose transporter; PKC, Protein kinase C; pp38MAPK, Mitogen-activated protein kinase; ERK1/2, Extracellular signal-regulated kinase 1/2; OGTT, Oral glucose tolerance test; STZ, Streptozotocin; MIN6, Mouse INsulinoma 6; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus.

* Corresponding author.
E-mail addresses: gurprit.bhardwaj@gmail.com, gurprit.bhardwaj-biochem@msubaroda.ac.in (G. Bhardwaj), sglmescrl@gmail.com (S. Gupta).

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commercially available SGLT2 inhibitors for the management of high glucose levels. Gliflozins like canagliflozin, dapagliflozin, empagliflozin inhibit the reabsorption of glucose in the kidney, thereby causing excretion of glucose in the urine (glucosuria) [4]. Apart from SGLT2 inhibition, other potential actions have also been attributed to this class of drugs. One of the SGLT2 inhibitors, empagliflozin treatment was found to be related to improved β-cell function in type 2 diabetic patients and preserving β-cell mass in type 1 diabetes in mice model studies [5,6]. On the other side, canagliflozin also improves β-cell function in patients with type 2 diabetes [7]. Hamamatsu et al. demonstrated that diabetic mice treated with canagliflozin significantly preserved β-cell mass [8]. So gliflozins have beneficial effects on pancreatic β-cells apart from inhibiting SGLT2. The important role of the natural compound phlorizin in the development of SGLT inhibitors has been extensively reported. Besides phlorizin, various flavonoids and especially flavonoid enriched plant extracts have been explored for their glucose lowering effect targeting SGLT2. Phlorizin and naturally available flavonoid glycosides have paved the way for the development of various C glycosides and their analogs [9–11].

One such bioactive, swertisin, from a medicinal plant *Enicostemma Littorale* (EL) has been explored as a potent islet neogenic agent by our group [12,13]. SGLT inhibitors have demonstrated protection against failing pancreas [14]. Swertisin being a C-glucosyl flavone has been explored for SGLT2 inhibition using the target prediction tool [15] and explored various metabolic alterations associated with the application of swertisin in both *in vitro* and *in vivo* diabetic models. We have elucidated the role of swertisin in establishing a link between its glucose lowering action and SGLT2 inhibition and proved it an excellent antidiabetic drug.

2. Methods

2.1. In silico studies

In silico target prediction of swertisin was done by swiss target prediction tool [15]. We then proceeded for homology modelling of SGLT2 using the I-TASSER server [16]. The model was evaluated by the Ramachandran Plot predicted by the Rampage server [17]. The coordinates for Swertisin (CID: 124034) and Canagliflozin (CID: 24812758) were downloaded from the PubChem database [18]. The ligands were minimized with CHARMM forcefield using Discovery Studio v.20 for 2000 max steps and with Momany-Rone partial charge estimation. The ligands (Swertisin and Canagliflozin) and receptor (hSGLT2) used for molecular docking were prepared using AutoDock Tools 4.2 graphical interface [19]. The PDB coordinates were converted to PDBQT format which includes added charges if necessary, merge non-polar hydrogens, and assign appropriate atom types. Affinity (grid) maps were generated by placing the center of the grid near active site residues. For molecular docking, the AutoDock Vina program [20] was used. The docked complexes were analysed using Discovery Studio Visualizer.

2.2. In vitro studies

2.2.1. Chemicals

Swertisin was previously isolated and stored from the whole dried plant of *Enicostemma littorale* as reported [12,13,21]. Canagliflozin was commercially purchased as INVOKANA® (Janssen Pharmaceuticals, Inc.).

2.2.2. Cell culture

HEK293 and Caco2 cell lines were propagated at 37 °C in 5% CO_2 in DMEM high glucose (Gibco#12100-046) supplemented with 1.0% of penicillin-streptomycin (Gibco#15140-122) and 10% FBS (Gibco#10270-106).

2.2.3. Sodium dependent glucose uptake assay

2.2.3.1. Fluorescence microplate analysis. 10,000 cells of HEK 293 or Caco2 were plated in 96-well plates. After 24 h preincubation, cells were washed with sodium free buffer (140 mM choline chloride,5 mM KCl, 2.5 mM $CaCl_2$,1 mM MgSO4, 1 mM KH2PO4, and 10 mM HEPES (pH 7.4, adjusted with 2.5 M Tris). Cells were serum starved and then preincubated with 0,7.5,15,30 and 40 μg/ml swertisin for 15 min to which was added 2-NBDG (Invitrogen#N13195) in sodium buffer (Sodium buffer contained 140 mM NaCl instead of choline chloride), sodium free buffer, and sodium buffer with 10 μM cytochalasin B (MP Biomedical#195119) (GLUT inhibitor) [22] for 60 min. After 60 min, the buffers were removed and the cells were rinsed in sodium free buffer and lysed with cold lysis buffer (1% Nonidet P-40,1% sodium deoxycholate, 40 mM KCl, 20 mM Tris, pH 7.4). The fluorescence intensity was detected on Biotek Synergy HT (USA) microplate reader (Excitation: 485/20, Emission: 528/20). To measure DNA, Hoechst (Himedia#TC266) was added and fluorescence intensity was measured (Excitation: 360/40, Emission: 460/40). 30 μM canagliflozin was taken as a positive control [23].

2.2.3.2. Fluorescence microscopic analysis. 0.5×10^6 cells of HEK293 cell line was seeded in 35 mm cell culture dish was grown about 80% confluent. Cells were washed with sodium free buffer thrice after aspiration of cell culture media. Cells were then preincubated for 15 min with and without 7.5 μg/ml swertisin in sodium buffer with 10 μM cytochalasin B. 100 μM of 2-NBDG was added and Live cell imaging was performed with "Objective Lens" "UPLSAPO 20X", "Objective Lens Mag."'"20.0X" and "Objective Lens NA"'"0.75" under a confocal microscope (FV3000 Olympus, USA) with incubation at 37 °C and 5% CO2. Cells without swertisin treatment were considered as control.

2.3. Protein extraction and western blotting

HEK293 cells or Kidney tissues were harvested and kept on ice. Minced tissue powder or harvested cell lysate were lysed in Laemmli lysis buffer. Total protein concentration was estimated by Bradford's method and 20 μg or 15 μg of total protein were resolved and transferred to nitrocellulose membrane for tissues and cells respectively. After blocking was performed for 1 h, blots were subsequently probed with SGLT2(Abcam#37296), PKC (Millipore#07-264), pp38MAPK (Cell Signalling#9216), ERK1/2 (Cell Signalling#9102), and Beta-actin (BD bioscience#612657) primary antibodies overnight at 4 °C. Blots were then incubated with respective secondary antibodies conjugated with HRP for 1 h at RT. Specific bands of proteins were visualized using enhanced chemiluminescence (ECL) reagent (Bio-Rad) and images were captured on Alliance 4.7 UVI Tec Chemidoc (Uvitech, Cambridge) gel documentation system. Densitometric analysis was carried out by Image J software.

2.4. In vivo studies

2.4.1. Animal selection and induction of diabetes and in vivo experimental design

6–8 weeks old adult male BALB/c mice were kept at the animal house with 12 h light and dark cycle with water and pellet diet *ad libitum*. These studies were carried out in strict accordance as per the guidelines and approval of the institutional Committee for the Purpose of Control and Supervision on Experiments on Animals, India (CPCSEA) (Protocol no. MSU/BC/IAEC/2016/04). After successfully inducing diabetes with STZ injection (65 mg/kg body weight) for 5 days, the Fasting Blood Glucose of animals was confirmed using Accu-check Performa glucometer (Accu-check, Roche, USA) at regular intervals to monitor their diabetic status. After establishing the STZ induced diabetic mice model, mice were divided into four groups control, diabetic, swertisin, and

canagliflozin treatment. Each group had 8 to 12 mice each. STZ diabetic mice were treated with swertisin (2.5 mg/kg body weight) and other group treated with canagliflozin (10 mg/kg body weight) from 0 day of experiment till the 15th day. Swertisin was administered with saline intraperitoneally. On the penultimate day of the study, mice from each experimental group were individually housed in metabolic cages by gradual acclimatization with 12 h light and dark cycle with water and pellet diet *ad libitum*. Data were collected in the morning (07:00 h). Animal weight, water, chow consumption, and urine volume were recorded and urine specimens were taken for analysis. Proteinuria was

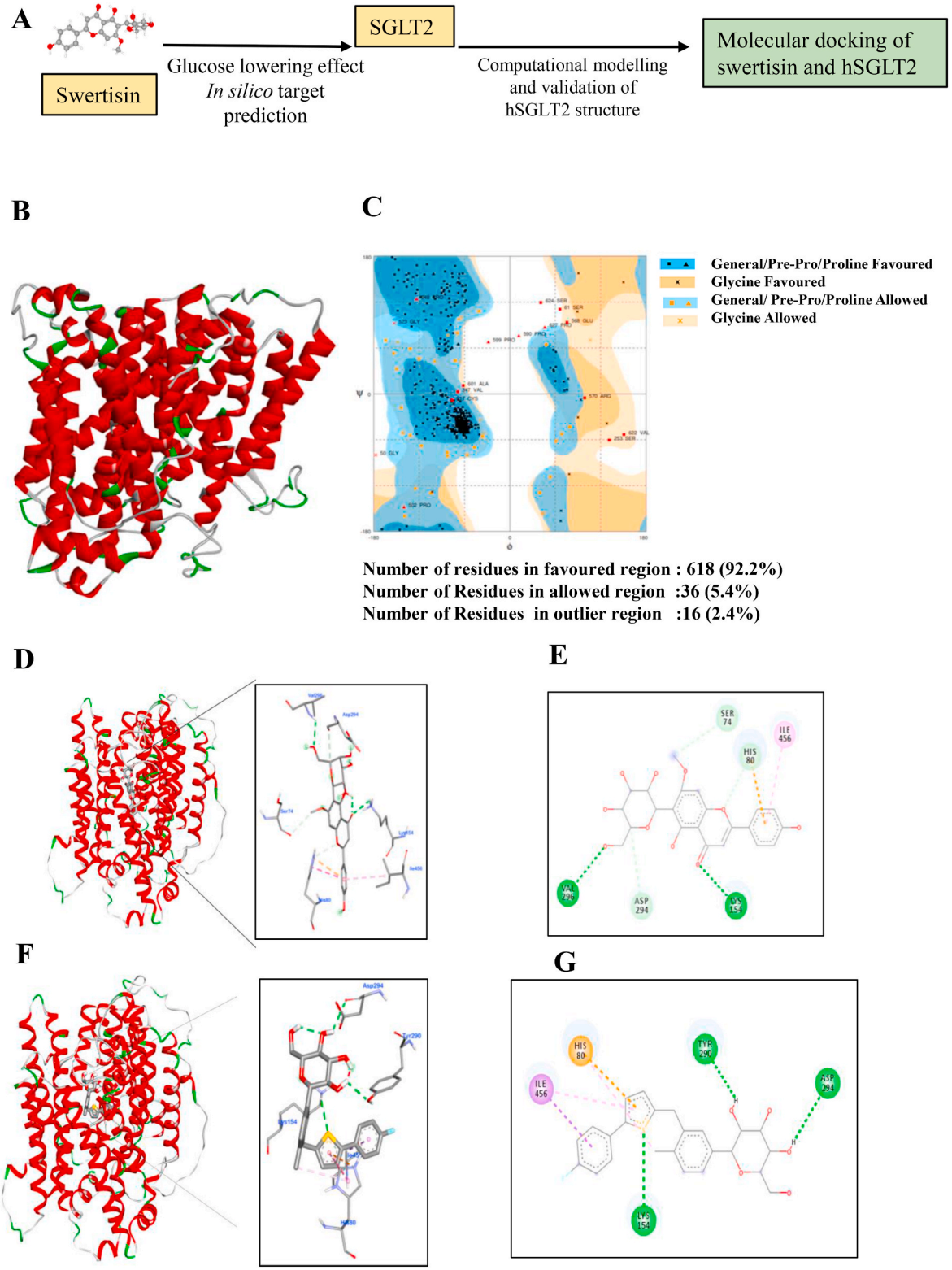

Fig. 1. Swertisin interacts with key residues within the active site of hSGLT2 by molecular docking. (A) *In silico* plan of work (B) Homology model of hSGLT2 (C) Ramachandran plot analysis of the constructed model (D–E)3D and 2D diagram of molecular docking interaction of Swertisin-hSGLT2 (F–G) 3D and 2D diagram of molecular docking interaction of Canagliflozin-hSGLT2.

analysed. Serum and urine samples were analysed for urea and creatinine using Reckon diagnostics P. LTD.(India) kits. For the endpoint oral glucose tolerance test, glucose tolerance was monitored by taking blood glucose reading at regular intervals until 2 h.

2.5. Statistical analysis

The data is represented as Mean ± SEM. The significance of difference was evaluated by the Student's t-test or ANOVA by Graphpad prism 7.

3. Results

3.1. Swertisin- SGLT2 docked complex explains SGLT2 inhibitory action of swertisin

Sodium glucose cotransporter 2 was one of the targets of swertisin by the target prediction tool. We used computational docking studies to understand structural complexities and binding affinities between the target (SGLT2) and ligand (swertisin). So, we computationally modelled the human SGLT2 structure and proceeded with molecular docking (Fig. 1A). The homology model of hSGLT2 constructed using I-TASSER was having an acceptable C-score of −0.68 [range: -2 to 5]. I-TASSER uses a multi-template threading approach and for construction of the hSGLT2 model, PDB: 3DH4 and PDB: 2XQ2 (Crystal structure of Na/Sugar symporter from *Vibrio parahaemolyticus* were used as major templates (Fig. 1B). The hSGLT2 model was evaluated using the Ramachandran Plot with 92.2% residues in the favoured region, 5.4% residues in the allowed region, and 2.4% residues in the outlier region (Fig. 1C). The minimized ligands had final potential energy of 39.62 from initial 90.90 kcal/mol for Swertisin and 20.72 from initial 62.21 kcal/mol for Canagliflozin. Affinity (grid) maps of 15.01 × 21.51 × 18.98 Å grid points with 29.48, −41.04, and 54.97 x, y, and z spacing respectively were generated for molecular docking covering important residues in the active site region. For molecular docking of Swertisin with hSGLT2, SGLT2 inhibitor canagliflozin was also used for docking studies. The docking score for the Swertisin-hSGLT2-interaction and Canagliflozin-hSGLT2-interaction were determined to be −8.5 and −8.7 kcal/mol respectively. In the case of Swertisin, S74, H80, K154, D294, and V296, formed h-bond with C15, O, O1, C10, and O3 atoms of swertisin respectively. The I456 and H80 of hSGLT2 formed a Pi-Alkyl and Pi-Cation interaction with the benzene ring of swertisin respectively (Fig. 1D and E). Two hydrogen atoms of canagliflozin (H9 & H11) formed hydrogen bonds with T290 and D294, whereas K154 formed an h-bond with S1 atom. H80 of hSGLT2 formed a Pi-Cation interaction with a five-carbon ring of canagliflozin and Pi-Alkyl interaction with C14 atom. The I456 of hSGLT2 formed a Pi-Sigma interaction with a benzene ring and Pi-Alkyl interaction with a five-carbon ring of canagliflozin (Fig. 1F and G). The overall interaction pattern indicates a stable interaction of swertisin within the active binding site of hSGLT2 which is also demonstrated by canagliflozin. Thus *in silico* data gives a similar activity of swertisin with canagliflozin suggesting swertisin a potential candidate for SGLT2 inhibitor.

3.2. Swertisin suppresses sodium dependent glucose uptake by selectively inhibiting SGLT2

SGLT2 and GLUT2 are major glucose transporters present in the kidney which facilitates glucose reabsorption in the blood which is sodium dependent and independent respectively [9]. Since the computational analysis affirmed the interaction of swertisin to that of SGLT2 which is mainly expressed in the kidney; *in vitro* investigation was performed to assess the sodium dependent and independent 2-NBDG uptake by swertisin in the HEK293 cell line. To probe whether swertisin interacts with GLUT transporter, uptake was performed in sodium free buffer. SGLT2 dependent uptake was again followed by sodium buffer with cytochalasin B respectively (Fig. 2A). Cytochalasin B is well characterized for inhibition of glucose transport by GLUTs [24,25]. It inhibited 2-NBDG uptake in pancreatic [26] and endothelial cells [27]. Some biochemical studies have demonstrated that cytochalasin B binds at or near to the sugar export site of GLUT1 in RBCs [28].

These tests revealed that 7.5 μg/ml concentration of swertisin strongly inhibited sodium dependent glucose uptake, reducing 2-NBDG from 100% to 51.4% and 30.4% in the absence or presence of cytochalasin B respectively compared to control. Uptake inhibition was consistent even at higher concentrations of swertisin (Fig. 2B and D). Whereas canagliflozin demonstrated reduced 2-NBDG by 34.36% from 100% at 13 μg/ml in sodium buffer with cytochalasin B compared to control. These results point out almost similar degree of glucose uptake inhibition by swertisin at a much lower dose than canagliflozin. As anticipated our sodium independent uptake of 2-NBDG experiment demonstrated that it was unaffected by swertisin (Fig. 2C).

Further, fluorescence microscopy analysis demonstrated time-dependent intracellular accumulation of 2- NBDG in HEK293 cells in control where it preferentially localizes inside the cell. Whereas striking aggregation of 2-NBDG on HEK293 cell membrane in swertisin treated cells was evident where 2-NBDG can be observed as confined to the HEK293 cell membrane (Fig. 2E).(See the supplemental material for live confocal imaging video). These results further strengthen our conviction about the SGLT2 inhibition property of swertisin.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ceramint.2019.04.220

To study the effect of swertisin on SGLT1, we performed similar sodium dependent and independent 2-NBDG uptake in Caco2 cell line (Supplementary Fig. 1 A-D). We observed unaltered and non-significant inhibition of glucose uptake even at a higher concentration of swertisin. Thus, swertisin displayed higher selectivity of SGLT2 rather than SGLT1 at lower inhibitory concentration compared to canagliflozin.

3.3. Swertisin regulates key SGLT2 related proteins

One of the contributing factors in uptake of glucose by the transporters in the cells depends on the expression of the transporter protein which in turn is regulated by many factors. So we wanted to determine whether swertisin contributed to the regulation of SGLT2 expression, we examined the time-dependent expression of SGLT2 in presence of swertisin. As expected, incubation of the HEK293 cell line with 7.5 μg/ml swertisin abolished the induction of SGLT2 expression in a time dependent manner (Fig. 3D). Evident downregulation of SGLT2 protein expression was persisted till 12 h incubation with swertisin compared to control.

It led us to explore the protein expression of kinases which contributes to the regulation of SGLT2. Stimulation of Protein kinase C is known to regulate SGLT2. pp38 MAPK and *Erk*1/2 also plays role in regulating the expression of the SGLT2 [2,3] In the present study, we observed downregulation of SGLT2 with the upregulation of PKC (Fig. 3A) from 4 h till 12 h of post swertisin treatment incubation.

SGLT2 regulation by PKC involves pp38 MAPK (Fig. 3B) which also demonstrated increased expression than control at 12 h. Another important kinase is ERK1/2 (Fig. 3C) and non-significant change was observed until 12 h. Thus, temporal analysis of SGLT2 levels with swertisin treatment demonstrated overall downregulation of SGLT2 (Fig. 3D) with differential regulation of regulating proteins.

3.4. Swertisin improves glycaemic control in STZ induced diabetic mice: an in vivo study

In silico and *in vitro* results established the association of swertisin to that of SGLT2. To get a deeper understanding of SGLT2 inhibition by swertisin, we then proceeded for *in vivo* model to get insight of swertisin SGLT2 inhibitory action at a physiological level by monitoring the efficacy of swertisin in STZ induced diabetic Balb/c mice. The effect of

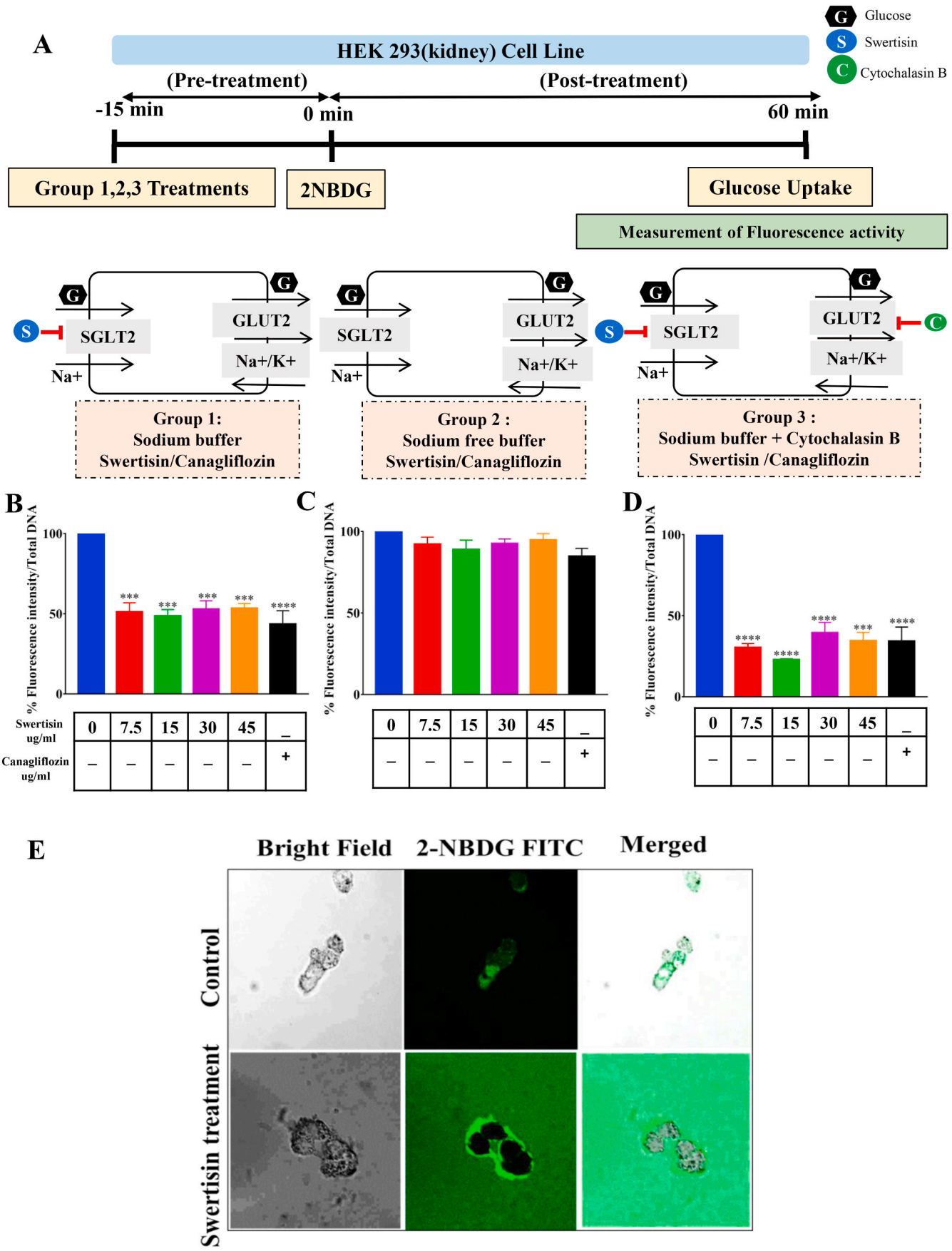

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Fig. 2. SGLT2 specific inhibition by swertisin affects sodium dependent glucose uptake. (A) *In vitro* plan of work. Sodium dependent glucose uptake assay was performed in the HEK293 cell line. Swertisin treatment was given at varying doses and uptake inhibition of 2-NBDG was performed in (B) sodium buffer (C) sodium free buffer and (D) sodium buffer with 10 μM cytochalasin B (GLUT inhibitor) for 60 min. Canagliflozin was taken as a positive control. Results are represented as % Fluorescence intensity per total DNA ± SEM, N = 3. Significance is expressed as p-value *** <0.001, **** <0.0001 control vs treatment groups. (E) Representative time dependent fluorescence imaging of uptake was performed HEK293 cells were incubated in sodium buffer in the absence (Control) and presence of 7.5 μg/ml swertisin with 10 μM cytochalasin B for 10 min in presence of 2-NBDG (green) (Magnification:20X) G = Glucose, S=Swertisin, C=Cytochalasin B. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article). (See the supplemental material for live confocal imaging video)

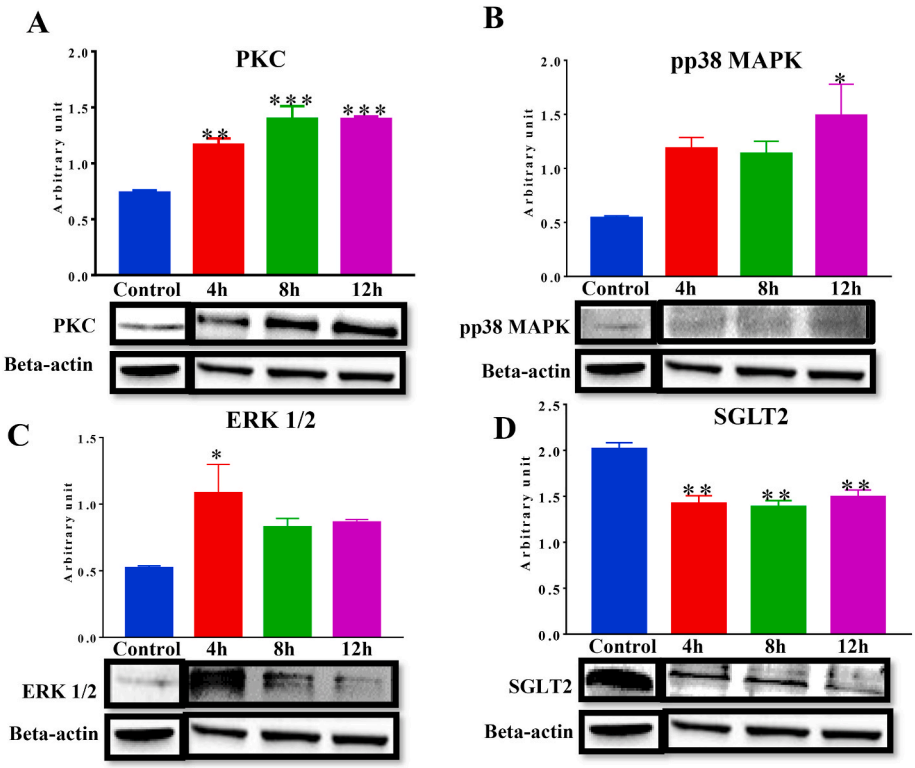

Fig. 3. Swertisin selectively regulates SGLT2 expression. Time dependent protein expression of SGLT2 and regulating factors in the HEK293 cell line were studied. Western Blot analysis of proteins (A) PKC, (B) pp38 MAPK (C) ERK1/2, and (D) SGLT2 along with densitometric analysis normalized to Beta-actin are expressed as arbitrary unit± S.E.M. N = 3, Significance is expressed as p-value *<0.05, ** <0.01, *** <0.001 control vs treatment groups.

swertisin and canagliflozin was monitored over 15 days in diabetic mice. Swertisin dose was initiated at day 0 (326 ± 46.11 mg/dl) of treatment and reduced levels of fasting blood glucose was evident right from day 5th of treatment (253 ± 30.72 mg/dl), day 10th (230 ± 61.75 mg/dl) and persisted till day 15th (124 ± 19.08 mg/dl) (Fig. 4A). Remarkably the extent of glucose lowering effect of swertisin was observed at a much lower dose (2.5 mg/kg body weight) compared to positive control drug canagliflozin (10 mg/kg body weight). These results prove a higher potency of swertisin than canagliflozin for demonstrating sustainable and consistent glucose lowering effect. An oral glucose tolerance test (OGTT) was performed on Day 15th on overnight fasted mice which demonstrated controlled glycemia over the period of 2 h (Fig. 4B). As predicted, OGTT results were also comparable with canagliflozin treated mice.

3.5. *Swertisin affects physiological and metabolic parameters in STZ induced diabetic mice*

In silico and *in vitro* results established the association of swertisin to that of SGLT2. We then examined several physiological parameters in all the treatment groups of mice. Inhibition of SGLT2 is expressed as changes in various metabolic parameters ultimately affecting glucose homeostasis. STZ induced weight loss in mice is commonly observed and was persistently detected in swertisin and canagliflozin treated mice (Fig. 5A). Chow intake of the Diabetic and Canagliflozin group was significantly increased compared to control mice (Fig. 5B). On the contrary, swertisin and canagliflozin groups demonstrated decreased water intake compared to the diabetic (Fig. 5C). Urine output was significantly higher in diabetic, swertisin, and canagliflozin groups with respect to control (Fig. 5D).

Glucosuria and proteinuria are a few of the important characteristics of diabetes [9,29]. Swertisin and the canagliflozin group demonstrated lesser proteinuria compared to a diabetic with the least proteinuria in the swertisin group (Fig. 5E). Glucosuria was higher in swertisin and canagliflozin groups compared to the diabetic group with the swertisin group showing the highest glucosuria (Fig. 5F).

SGLT2 inhibition affects renal functions and pertains to intrarenal and extrarenal effects [4]. Serum (Fig. 5G) and urine (Fig. 5H) creatinine levels were increased in diabetic mice compared to control mice. Creatinine clearance (Fig. 5I) was higher in a diabetic with respect to control mice. Serum urea decreased in swertisin and canagliflozin treatment compared to the diabetic group (Fig. 5J). Urine urea increased in swertisin treatment compared to the diabetic group (Fig. 5K). Urea clearance increased in swertisin treatment and canagliflozin treatment concerning diabetic (Fig. 5M).

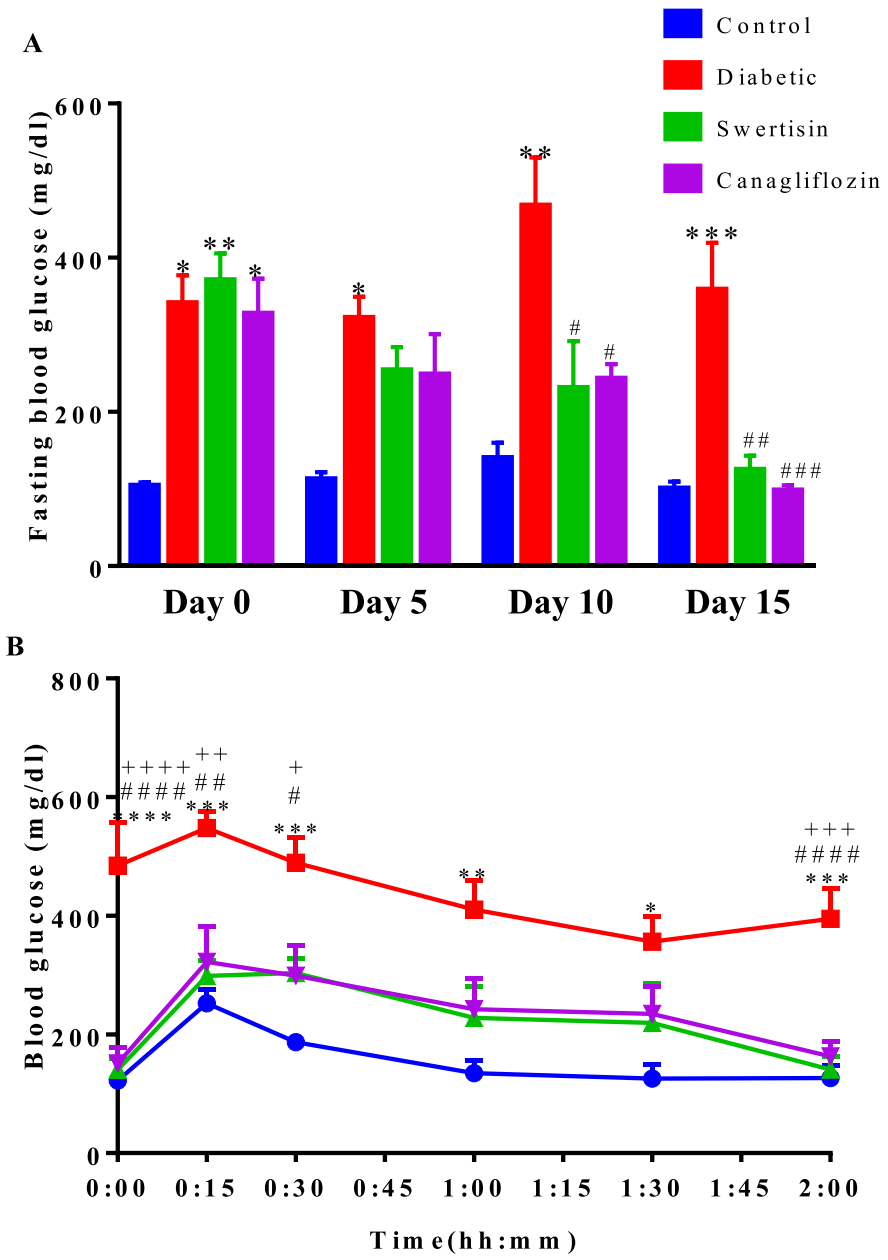

Fig. 4. Swertisin ameliorates glycaemic control in STZ induced diabetic mice. (A) Graph representing fasting blood glucose at different days of treatment for control, diabetic, swertisin and canagliflozin treated STZ diabetic BALB/c mice groups. Data are represented as mean ± SEM. *<0.05, ** <0.01, *** <0.001 Control vs treatment groups #<0.05, ## <0.01, ### <0.01 Diabetic vs treatment groups (N = 8-12) (B) Graph representing blood glucose levels for oral glucose tolerance test over 2 h for control, diabetic, swertisin and canagliflozin treated STZ diabetic BALB/c mice. Data are represented as mean ± SEM. *<0.05, ** <0.01, *** <0.001, **** <0.0001 Diabetic vs control, #<0.05, ## <0.01, #### <0.0001 Diabetic vs swertisin treatment. Diabetic vs canagliflozin treatment +<0.05, ++ <0.01, +++ <0.001, ++++ <0.0001 (N = 8).

3.6. Swertisin reduces expression of SGLT2 along with inhibition in kidney tissue of mice

Reduction in SGLT2 expression in diabetic condition has been demonstrated to improve the overall glucose homeostasis of the body by reducing the reabsorption of glucose in the blood by the kidney [30]. We next addressed the question of whether swertisin affects the expression of SGLT2 in the kidney. After 15 days of treatment increased expression of SGLT2 in the diabetic and canagliflozin treated group were observed compared to control. However, the most striking result to emerge from the data is the critically reduced expression of SGLT2 which was observed in the swertisin group as compared to diabetic and canagliflozin treated mice (Fig. 6B). We also assessed PKC expression under swertisin action and favorably found that swertisin treatment not only reduced SGLT2 expression but also reduced PKC expression as well compared to diabetic (Fig. 6A). PKC was found upregulated in diabetic, swertisin, and canagliflozin groups. PKC was also found upregulated in the canagliflozin group compared to diabetic and swertisin groups. These results offer indisputable evidence that swertisin action involves the reduction in SGLT2 expression along with inhibition of SGLT2 action which clearly shows an advantage over canagliflozin which only inhibits the action of SGLT2 with unaltered SGLT2 expression.

4. Discussion

The primary goal of this study was to establish the glucose lowering action of swertisin to its SGLT2 inhibition property. Our *in silico* data demonstrated stable molecular binding of swertisin with SGLT2 by molecular docking. Marketed SGLT2 inhibitor canagliflozin served as a comparator for our studies [31]. Appreciably, the docking scores for Canagliflozin-hSGLT2 (−8.7 kcal/mol) and Swertisin-hSGLT2-interactions (−8.5 kcal/mol) were similar. This result strongly pointed to the likelihood of steady binding of swertisin with SGLT2.

Asp 294 of SGLT-2 is important for sugar-binding. Both luteolin and orientin, bioactive flavonoids, indicated *in silico* interaction with Asp 294 [32]. Similarly, our structural docking study demonstrated that C10

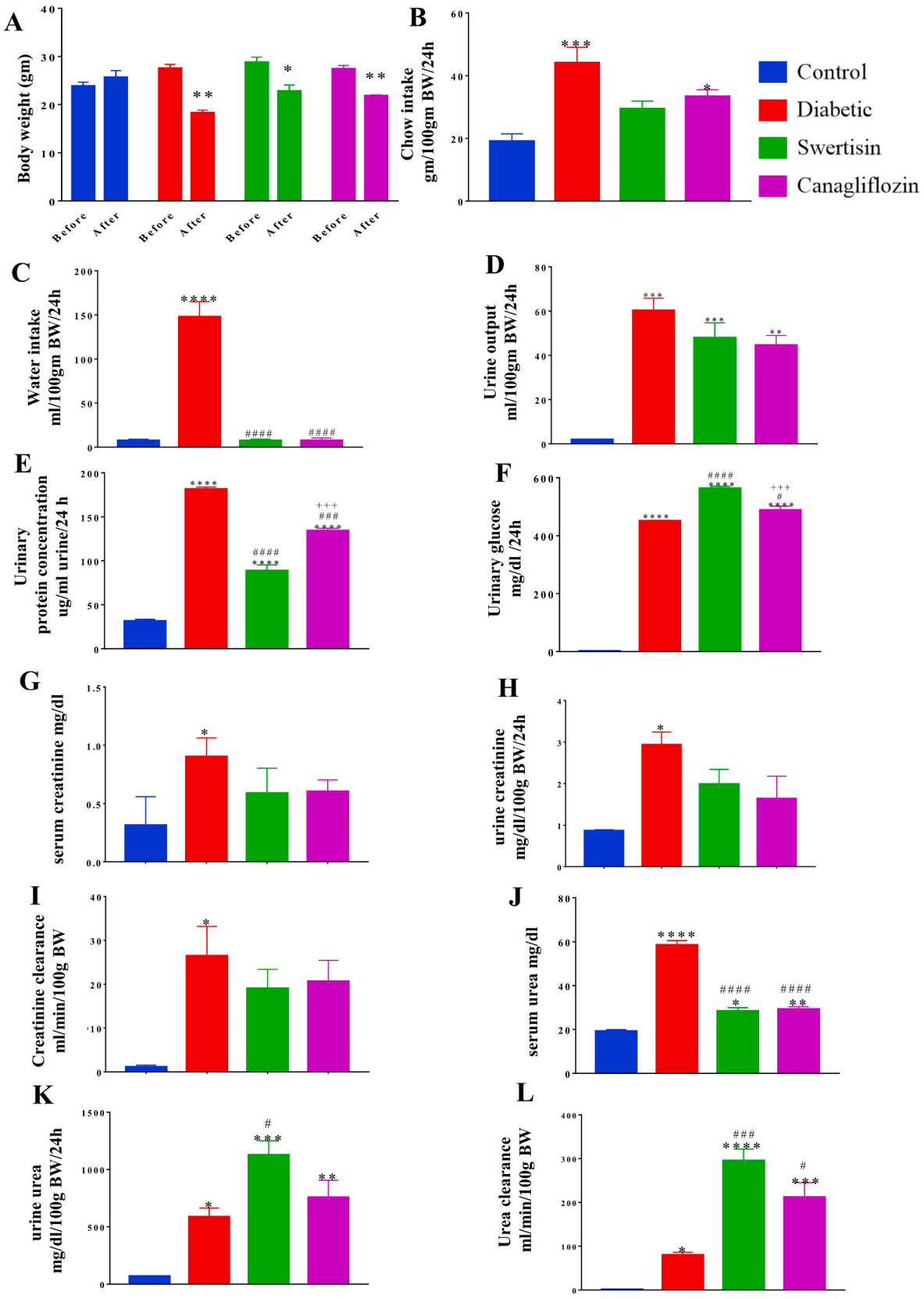

Fig. 5. Swertisin impacts physiological and metabolic parameters in STZ induced diabetic mice. Graphs representing different parameters (A) Body weight (B) Chow intake (C) water intake (D) urine output (E) proteinuria (F) glucosuria (G) serum creatinine (H) urine creatinine (I) creatinine clearance (J) serum urea (K) urine urea (L) urea clearance for control, diabetic control, swertisin and canagliflozin treated STZ diabetic BALB/c mice groups. Data are represented as mean ± SEM. *<0.05, ** <0.01, *** <0.001, **** <0.0001 Control vs treatment groups #<0.05, ### <0.001, #### <0.0001 Diabetic control vs treatment groups. Swertisin treatment vs canagliflozin treatment +++ <0.001 (N = 8).

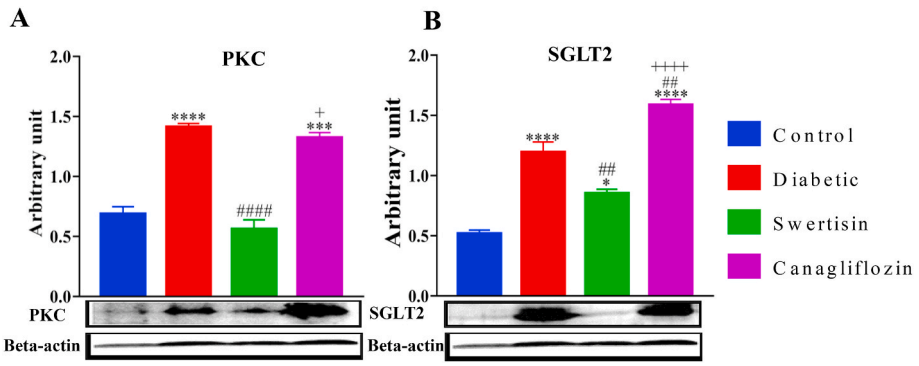

Fig. 6. SGLT2 and PKC expression is reduced by swertisin in mice kidney. Western Blot analysis of proteins PKC and SGLT2 along with densitometric analysis normalized to respective internal control Beta-actin Data are represented as mean ± SEM. p *<0.05, *** <0.001, **** <0.0001 Control vs treatment groups ## <0.01, #### <0.001 Diabetic control vs treatment groups. Swertisin treatment vs canagliflozin treatment +<0.05, ++++ <0.0001 (N = 3).

atom of swertisin formed h-bond with Asp 294 which was a conserved pattern followed by canagliflozin as well.

The marked observation that emerged from our *in vitro* sodium dependent glucose uptake experiment was that the potency of SGLT2 inhibition by swertisin was significantly effective at a lower dose as against canagliflozin.

The selectivity for SGLT was confirmed by no inhibition of glucose uptake by swertisin in sodium free buffer. Inhibition of GLUT transporters leads to undesirable and adverse consequences due to the central key role of GLUT transporters in glucose homeostasis and metabolism [10] Our findings confirm that swertisin does not interfere with non-sodium dependent GLUT transporters and further substantiates the selective inhibition for SGLT2 as against GLUT2 transporter.

In agreement with uptake results, aggregation of 2-NBDG outside the cell membrane of the kidney cell line critically confirms inhibition of SGLT2 by swertisin. This observation correlates favorably well with Ishihara et al. who reported the uptake of 3-*O*-methyl-D-glucose which is rapid and equilibration is 80% complete in 1 min in MIN6 cells [33].

Further, additional support for the selectivity of swertisin was demonstrated using the Caco2 (intestinal) cell line having SGLT1 as a major glucose transporter. SGLT1 inhibition can result in gastrointestinal side effects such as dehydration, diarrhea, and malabsorption since it is mainly expressed in the small intestine and helps in the absorption of glucose and galactose [34]. As expected, we observed a non-significant change in sodium dependent glucose uptake even at higher doses of swertisin. This can be highly attributed to the C glycosylation of the swertisin structure. Compared to phlorizin and *O*-glucosyl analogs, C-glycosylation enables achieving higher selectivity for SGLT2 over SGLT1 and GLUT [10]. Panchapakesan et al. reported that SGLT1 and GLUT2 expression are unaffected by hyperglycemia and also unaffected by inhibition of SGLT2 [35].

Further we investigated regulation of SGLT2 expression under the effect of swertisin with differential expression of some protein kinases. An important observation was the reduced expression of SGLT2 protein. PKC and MAPK pathways play a major role in regulating SGLT2 under hyperglycaemic conditions [3]. Upregulated PKC expression is often correlated with upregulated SGLT2 expression [1]. Although hyperglycemia-induced high expression of PKC was observed by swertisin treatment with downregulation of SGLT2 in *in vitro* study, it can be interpreted that swertisin does not affect PKC expression in acute treatment duration. Similarly, the expression of pp38MAPK and ERK1/2 was found to be higher. Haneda et al. lend support to high levels of ERK via hyperglycemia-induced PKC in the HEK293 cell line [2] which summarizes the differential regulation of PKC-MAPK pathway in the kidney by swertisin.

We then moved to a pre-clinical *in vivo* animal study with some inexplicable insights. Observation of a striking reduction in fasting blood glucose of STZ treated mice by swertisin treatment substantiated our findings of improved glycaemic index. This is in good agreement with Liang et al. where canagliflozin improved glycaemic control in terms of blood glucose. Canagliflozin has also reported improved oral glucose tolerance test [31] which was evident for swertisin. Our finding highlights the fact that the improvement in glycaemic control by swertisin is strikingly at par with canagliflozin.

Body parameters are important for evaluating diabetogenic and metabolic parameters. Bodyweight loss in STZ induced swertisin treatment group was remarkably evident before and after treatment. Further weight loss is also aided by canagliflozin. Ji et al. further reported canagliflozin treatment increases body weight loss in diabetic mice [36]. A similar contribution of swertisin for weight management is noteworthy in our *in vivo* study.

Chow intake did not alter significantly in the swertisin group compared to the diabetic but intake was higher in the canagliflozin treated group compared to control which is also evident by Matsuba et al. who also observed that despite the increase of the calorie intake, weight loss was evident with better glycaemic control [37]. Water intake was significantly lowered but an increase in urine volume in swertisin and canagliflozin groups compared to diabetic was observed as a contrast to Tanaka et al. who found that canagliflozin did not significantly inhibit water intake but also observed increase in urine volume as exhibited by diabetic patients [38]. The most striking observation of SGLT2 inhibition is glucosuria along with proteinuria which correlates favorably well with reports [9,29]. Though there were differences among the control and diabetic group, the creatinine and urea levels were within normal physiological range and thus rule out the possibility of kidney dysfunction so to generate a strong possibility of effects emerging from exclusive SGLT2 inhibition which is the major focus of the study and not from kidney damage which is usually seen in chronic diabetic nephropathy models [29].

Renal SGLT2 expression was found upregulated in untreated diabetes in humans as well as T1DM and T2DM murine models. Higher PKC expression is found in the kidney in diabetes [1]. Rahmoune et al. have also observed high glucose uptake and upregulated SGLT2 expression in diabetic patients [30].

The most remarkable observation to emerge from these data was that swertisin was able to downregulate the protein expression of SGLT2 in contrast to canagliflozin. This is in good agreement with Maki et al. where they found that high glucose-induced increased expression of SGLT2 was not affected significantly by canagliflozin [39]. On the contrary, swertisin reduced SGLT2 expression which makes swertisin a better option than canagliflozin in diabetes therapeutics.

Diabetes being a multiorgan multitarget metabolic syndrome encompasses various receptors, transporters and proteins which play a key role in management of diabetes [40]. Having demonstrated the functional property of swertisin as SGLT2 inhibitor, another interesting aspect of SGLT2 as demonstrated by Lee et al. is its antagonistic role to

adenosine A1 receptor (A1AR). They demonstrated the ameliorating effects of swertisin on scopolamine-induced memory impairment via the involvement of adenosine (ADO)/A1AR signalling [41] This signalling also contributes to insulin-controlled glucose homeostasis [42]. Though to delimit the scope of current study, cross talk of A1AR and SGLT2 inhibitor has not been addressed, but it would be intriguing and important to document link between swertisin, ADO/A1AR signalling and improvement in glucose homeostasis as future prospects.

5. Conclusion

Based on *in silico, in vitro,* and *in vivo* studies our research has highlighted the importance of SGLT2 inhibition and its reduced expression by swertisin. The direct role of swertisin in controlling hyperglycemia makes it an excellent pharmacophore agent that can ease the burden of diabetes healthcare management by providing holistic treatment and a foremost candidate for SGLT2 inhibitors.

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Authors contribution

GB Conceptualization, Methodology, Investigation, Software, Data curation, Formal analysis, Writing- Original draft preparation **MV** Supervision, Methodology, Validation, Reviewing and Editing **AS** Supervision, Methodology **DP**, **AP, PM, RS** software and *in silico*, **HS** Methodology, Investigation, Data curation **SG** Conceptualization, Visualization, and Supervision.

Declarations of competing interest

The Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2021.108995.

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Research article

Influence of metabolically compromised Adipose derived stem cell secretome on islet differentiation and functionality

Gurprit Bhardwaj [a], Mitul Vakani [a], Abhay Srivastava [b], Komal Rawal [a], Amrita Kalathil [a], Sarita Gupta [a,*]

[a] Molecular Endocrinology and Stem Cell Research Laboratory, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, 390021, India
[b] Regenerative Medicine Program, Institute of Cardiovascular Sciences, Department of Physiology and Pathophysiology, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, MB, Canada

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ABSTRACT

Islet integrity plays a major role in maintaining glucose homeostasis and thus replenishment of damaged islets by differentiation of resident endocrine progenitors into neo islets regulates the islet functionality. Islet differentiation is affected by many factors including crosstalk with various organs by secretome. Adipose derived stem cells (ADSC) secrete a large array of factors in the extracellular milieu that exhibit regulatory effects on other tissues including pancreatic islets. The microenvironment of metabolically compromised human ADSCs (hADSCs) has a detrimental impact on islet functionality. In the present study, the role of secretome was studied on the differentiation of islets. Expression of key transcription factors like HNF–3B, NGN-3, NeuroD, PDX- 1, Maf-A, and GLUT-2 involved in development were differentially regulated in obese hADSC secretome as compared to control hADSC secretome. Islet like cell clusters (ILCCs) functionality and viability were critically hampered under obese hADSC secretome with compromised yield, morphometry, lower expression of C-peptide and Glucagon as well as higher ROS activity and cell death parameters. This study provides considerable insights on two major findings which are (i) exploring the use of hADSC secretome in islet differentiation and (ii) understanding the regulating effect of altered hADSC secretome under a metabolically compromised condition.

1. Introduction

Loss of functional pancreatic β cells is the underlying cause of type 1 diabetes mellitus which leads to multiple long-term complications. The critical contributor to this pathology is the autoimmune attack on functional beta cells. Apart from exogenous insulin treatment, current therapies include islet transplantation from cadaveric donors. But owing to exceeding demands placed on organ transplantation, there is still considerable uncertainty of the availability of donors, rejection post transplantation, and the need for immuno-suppressant. Thus, transplantation therapy demonstrates viable but still with multiple hurdles to be a successful therapeutic regimen [1]. This lead to the examination of the *in vitro* regeneration of β cells from various stem cell sources that can provide a sustainable solution for combating the need as a source for islet [2].

Stem cell therapy has gained wider recognition for its remarkable ability to support cell survival and homing capacity to the damaged tissue [3]. Islet differentiation can be modulated by various factors especially the milieu of secreted factors by other cells, which can vary in normal as well as pathological conditions. One such remarkable element is the regenerative capacity of secreted factors from human adipose tissue-derived stem cells (hADSCs). hADSC secretome is known to orchestrate various functions like wound healing, angiogenesis, anti-inflammation, immunomodulatory, anti apoptosis etc [4].

Conditioned media (CM) from stem cells have garnered much attention for regenerative potential without the involvement of the cell itself. It has also proved to have a beneficial effect on islet functionality. Various reports suggest direct or indirect co-culture of hADSC with islet having anti apoptotic effect and increased insulin secretion [5,6]. Nevertheless, the application of hADSC secretome on islet differentiation is yet to be properly defined. Also, if this secretome of hADSCs can be used as an *in vivo* differentiation agent for the resident PREPs, then it can be a better alternative to transplantation. It will exclude the risk of cell rejection and it is easy for packaging, transportation, and freeze

* Corresponding author.
E-mail addresses: gurprit.bhardwaj@gmail.com (G. Bhardwaj), sglmescrl@gmail.com (S. Gupta).

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drying [7].

On the other side, the secretome of hADSC has been shown to alter [8] under metabolically compromised conditions since the stem cell themselves are affected by a harsh micro environment [9]. Our lab has reported that obesity affects the metabolic profile of hADSC in the Indian population so we have also analyzed the modulatory effect of obese hADSC secretome on islet differentiation [10]. Further our lab has already explored the shortest route of islet differentiation from pancreatic resident endocrine progenitors (PREPs) and differentiated them into islet like cell clusters (ILCCs) with the help of islet differentiating agent 'swertisin' [11]. Thus, this research focuses on two major studies (i) Exploring the use of hADSC secretome in islet differentiation as a tool for regenerative medicine and (ii) Understanding the regulatory effect of altered hADSC secretome under a metabolically compromised condition on islet differentiation and functionality which is the first *in vitro* report to best of our knowledge.

2. Material and methods

2.1. Chemicals

Swertisin was previously yielded and stored from the whole dried plant of *Enicostemma littorale* as reported [12–15].

2.2. Cell culture

Human Adipose derived stem cells were previously isolated and cryopreserved from lipoaspirates from control (BMI≤23) and obese (BMI≥25) individuals as described earlier. Human ethical approval was obtained from Banker's Heart Institute (A Human Ethical and Central Drugs Standard Control Organization (CDSCO)) approved institute (ECR/214/Inst/Guj/2013/RR-16) and Institutional ethical committee (IECHR 2016-6), Vadodara, India [10,16]. hADSC were propagated at 37 °C in 5% CO2 in KnockOut™ DMEM (Gibco#10829-018) supplemented with 1.0% of penicillin-streptomycin (Gibco#15140-122) and 10% FBS (Gibco#10270-106).

Mouse pancreatic resident endocrine progenitors were previously isolated from a fresh islet and cryopreserved by digestion of mouse pancreas as described earlier [13,14,17] and were propagated at 37 °C in 5% CO2 in DMEM high glucose (Gibco#12100-046) supplemented with 1.0% of penicillin-streptomycin (Gibco#15140-122) and 10% FBS (Gibco#10270-106).

2.3. Conditioned media (CM) preparation from hADSC

hADSC were seeded until 80% confluency with KnockOut™ DMEM (Gibco#10829-018) with 10% FBS. After that culture media was replaced with serum free KnockOut™ DMEM and propagated at 37 °C in 5% CO2.After 24 h of incubation, media was collected centrifuged at 4000 rpm for 30min. Supernatant was stored at −80 °C after filtering through 0.2 mm filter until further experiment use.

2.4. LCMS of conditioned media (CM) of hADSC

LCMS analysis of secretome was performed on Q-Exactive Plus Biopharma-High Resolution Orbitrap Liquid Chromatograph Mass Spectrometer (Thermo Fischer Scientific Ptv. Ltd) (Facility hired at SAIF, IIT Bombay, India) The Chromatographic separation was performed on Analytical Column: PepMap RSLC C18 2 μm, 100A × 50 cm, Precolumn: Acclaim PepMap 100, 100 μm x 2 cm nanoviper. The mobile Phase used was: solvent A: 0.1% Formic acid in milliq water, solvent B: 80:20 (Acetonitrile:milliq water) + 0.1% Formic acid. Data acquitions and mass spectrometric data analysis software used was Thermo Proteome Discoverer 2.2. Abundance and classification of proteins were analyzed using Panther database [18].

2.5. Differentiation of ILCCs from PREPs under the effect of hADSC secretome

The PREPs were differentiated into ILCCs in presence of differentiation media which consists of serum-free KnockOut™ DMEM (Gibco#10829-018) media supplemented with 1% BSA, 1X glutamax,1X Zinc acetate, 1.0% of penicillin-streptomycin, insulin (5 μg/ml), transferrin (5 μg/ml), and selenite (5 ng/ml) cocktail as previously reported. PREPs were differentiated using swertisin (15 μg/ml) as differentiating agent [12–14]. PREPs were subjected to fifty percent of control and obese hADSC CM diluted with differentiation media till the end of islet differentiation in a 4 day protocol.

2.6. Yield, morphometry analysis and DTZ staining

ILCCs treated with control and obese hADSC CM were observed in a phase-contrast microscope (Nikon Instruments Inc.) and photographed after centrifuging and washing three times with PBS and then stained with DTZ solution. Evaluation of total yield was done and Morphometry analysis of ILCCs was performed using Image J software.

2.7. Immunocytochemistry

Differentiated ILCCs were collected, washed with PBS and centrifuged before fixing in chilled absolute methanol for 10 min. ILCCs were permeabilized with 0.1% Triton X-100 for 5 min at 4 °C. ILCCs were then subjected to blocking buffer (2% BSA) for 1 h at room temperature followed by incubation in C-peptide (CST#4593), glucagon (Sigma#G2654) primary antibodies overnight at 4 °C. ILCCs were washed and then incubated with respective secondary antibodies Anti-Rabbit-IgG-FITC (Sigma#F9887) and Anti-Mouse-IgG-CF555 (Sigma#-SAB4600299) for 1 h and then counterstained with DAPI, washed with PBS, mounted with coverslips. ILCCs were viewed under a confocal microscope (Zeiss LSM 710).

2.8. DCFDA staining

ILCCs were briefly centrifuged and washed with PBS. ILCCs were resuspended in 990 μl of PBS and 10 μl of 10 mg/ml of DCFDA (2',7'–dichlorofluoresceindiacetate) dye was then added and incubated at room temperature for 20 min. ILCCs were further incubated with DAPI for 10 min. ILCCs were centrifuged and washed with PBS. ILCCs were then observed under a phase contrast fluorescent microscope (Nikon Instruments Inc.).

2.9. FDA/PI staining

ILCCs were centrifuged washed with PBS. After that ILCCs were resuspended in PBS and FDA (0.5 mg/mL) and PI (2 mg/mL) were added to the ILCCs. After incubation at room temperature for 30 min, ILCCs were viewed under a phase contrast fluorescent microscope (Nikon Instruments Inc.).

2.10. Protein extraction and western blotting

ILCCs were centrifuged, washed, harvested and kept on ice. ILCCs were lysed in Laemmli lysis buffer. After that protein concentration was estimated by Bradford's method. 15 μg of protein were resolved and transferred to nitrocellulose membrane. Blots were then placed in blocking buffer for 1 h.Blots were then probed with Caspase-3 (Invitrogen#PA1-29157),Parp-1(Santacruz#SC-1561),HNF3–B (DSHB#4C7),NGN-3(Sigma#SAB1306585),NeuroD1(CST#4373),PDX-1(BD#554655),GLUT-2(Sigma #SAB1303865),MAF-A (Sigma #SAB2105099) and Beta-Actin (BD#612657) primary antibodies overnight at 4 °C. Blots were washed with PBST and PBS and incubated with respective HRP- conjugated secondary antibodies for 1 h at RT. Proteins

bands were visualized using enhanced chemiluminescence (ECL) reagent (Bio-Rad) and images were taken on Alliance 4.7 UVI Tec Chemidoc (Uvitech, Cambridge) gel documentation system. Densitometric analysis was done by Image J software.

2.11. Statistical analysis

Data is represented as Mean ± SEM and was evaluated by the Student's t-test by Graphpad prism 7.

3. Results

To study the crosstalk between hADSC and islet via trophic effects, we selected the shortest route of islet differentiation from pancreatic resident endocrine progenitors for studying the effects on islet differentiation and functionality. We investigated the effect of hADSC secretome of control and obese under normal and pathological conditions to analyze the effect on islet pathobiology.

3.1. Characterization of proteins of hADSC secretome from control and obese

To enhance our understanding of the characterization of different secretory products from the conditioned media of hADSC from control and obese individuals, conditioned media was subjected to LCMS analysis. A noteworthy difference in chromatogram was observed for both the secretome samples which substantiates the finding of altered secretome (Fig. 1A). A varying pattern of peaks was observed at different retention times. The highest chromatographic peak was observed at 48.10 min and 48.28 min in conditioned media of hADSC from control and obese respectively. Intriguing correlation is with the abundance of proteins which demonstrated differential expression of proteins in conditioned media of hADSC with a majority of the proteins overexpressing in obese (Fig. 1B). We categorized these proteins using gene ontology analysis (Panther database [18]) for their functional classification into molecular function, biological process, cellular component, protein class, and pathway. When analyzed for molecular function, most of the proteins considerably fall under catalytic activity (GO:0003824) followed by binding (GO:0005488) and molecular function regulator (GO:0098772). Biological processes categorized proteins majorly into the cellular process (GO:0009987) followed by metabolic process (GO:0008152), biological regulation (GO:0065007) and localization (GO:0051179). Cellular component classified proteins evidently for Cellular anatomical entity (GO:0110165) and intracellular (GO:0005622). Proteins were categorized under protein classes as transfer/carrier protein (PC00219), defense/immunity protein (PC00090), chromatin/chromatin-binding, or -regulatory protein (PC00077), translational protein (PC00263) protein-binding activity modulator (PC00095) with maximum proteins in metabolite interconversion enzyme (PC00262) category. For pathway analysis proteins were classified into Wnt signaling pathway (P00057), Nicotinic acetylcholine receptor signaling pathway (P00044), FAS signaling pathway (P00020), Inflammation mediated by chemokine and cytokine signaling pathway (P00031), Cytoskeletal regulation by Rho GTPase (P00016), Blood coagulation (P00011). These findings reinforce that there is an appreciable difference in the secretome which is an effect seen of subjugation of the hADSC to metabolically compromised environment.

3.2. ILCCs from PREPs under control hADSC secretome demonstrates optimal functional and phenotypic characterization

Owing to the fundamental interest in analyzing islet differentiation under the effect of secretory products of hADSC, PREPs were allowed to differentiate with control and obese hADSC secretome in a 4 day protocol supplemented with islet differentiation media combined with secretomes. Cluster formation was monitored microscopically for 4 days and more participation of PREPs, maximum cell aggregation, and zone of activation was observed in ILCCs under the effect of control hADSC secretome as a contrast to obese pointing effectively towards endocrine reprogramming (Fig. 2A). We further moved for confirmation of the maturation of ILCCs. DTZ stains insulin positive cells as it binds to zinc present in insulin granules and presents a crimson colour. A brighter red colour was observed, as zinc sequestration was present within insulin positive granules for ILCCs under the effect of control hADSC secretome as contrast to obese (Fig. 2B). For further considerable insight into the maturation of ILCCs, we immunostained the clusters with C peptide and glucagon. It was noteworthy to observe intense red and green staining in ILCCs under the effect of control hADSC secretome as contrast to obese confirming more insulin positive content. The critical correlation of immunocytochemistry of ILCCs pointed towards incomplete and immature functional differentiation under obese hADSC secretome (Fig. 2E). Further, we counted the ILCCs in both groups and observed more yield in the obese hADSC secretome group (Fig. 2C). Most importantly though the yield was higher, the population of ILCCs was heterogeneous with most ILCCs falling in 100–250 μm range and were scattered with loose edges in obese hADSC secretome whereas more compact and spherical were found in control (Fig. 2D). These results offered compelling evidence of the inadequacy of functional maturation of ILCCs when reinforced with secretory products of obese hADSC secretome.

3.3. ILCCs from PREPs under control hADSC secretome demonstrates high islet cell survival and integrity

The discrepancies in islet functionality led our investigation to estimate the levels of intracellular ROS generation of ILCCs in both groups. So we performed DCFDA staining which shows the intensity of intracellular oxidative stress. ILCCs with low green intensity were observed with control hADSC secretome as contrast to obese with higher green fluorescent intensity, suggesting the evident potential of control secretome for cytoprotection against ROS generation as compared to increased levels of intracellular oxidative stress in obese secretome (Fig. 3A).

To substantiate further findings, we evaluated the viability of ILCCs by FDA/PI staining. ILCCs with control hADSC secretome were observed to have more green fluorescent intensity compared to obese which suggests more viable cells since FDA stains live cells shown by green fluorescence. While dead cells are stained red by PI dye which was more evident in ILCCs with obese hADSC secretome. (Fig. 3B). This finding pointed towards the conviction of discordance of islet pathobiology under normal and pathological conditions.

Further cell death analysis was carried which correlated favorably with our findings. Cleaved caspase-3 and Parp1 (Fig. 3C) are reliable markers for cell death [19,20]. Inflammatory stressors, intracellular ROS, and oxidative stress cause DNA damage which activates caspase-3 (apoptotic marker) and Parp1 (DNA damage marker) and are hence associated with cell death. Within 4 day protocol of islet differentiation, we analyzed their expression on day 0, day 2, and day 4. We observed higher expression of both the markers on day 4 indicating ascend of ILCCs into cell death. Densitometric analysis of cleaved PARP1 and cleaved caspase 3 demonstrated increased expression compared to PREPs at day 0 as suggested for the fate of differentiation and molecular rearrangement of participating and non participating PREPs into cluster formation to yield viable and functional islet. This result substantiates the finding of altered islet biology under different secretome conditions.

3.4. ILCCs from PREPs under control hADSC secretome demonstrates high fidelity towards differentiation

To understand the implications of the fate of islet differentiation under the effect of control and obese hADSC secretome, we performed immunoblotting of key transcription factors which regulate islet

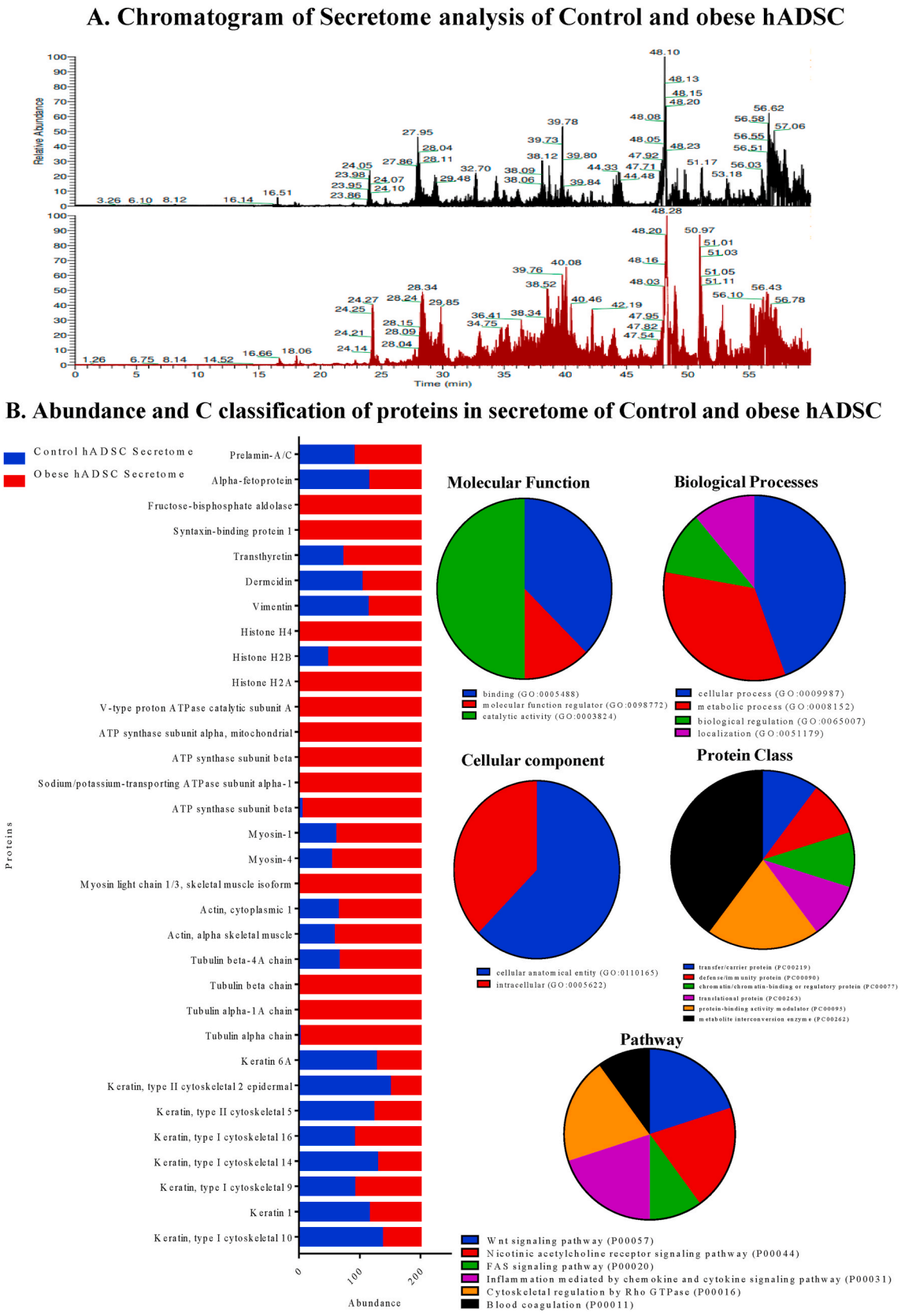

Fig. 1. Differential characterization of protein from secretomes (A) Chromatogram of secretomes of hADSC from control and obese (B) Graph showing abundance and (C) classification of proteins present in secretomes of hADSC from control and obese.

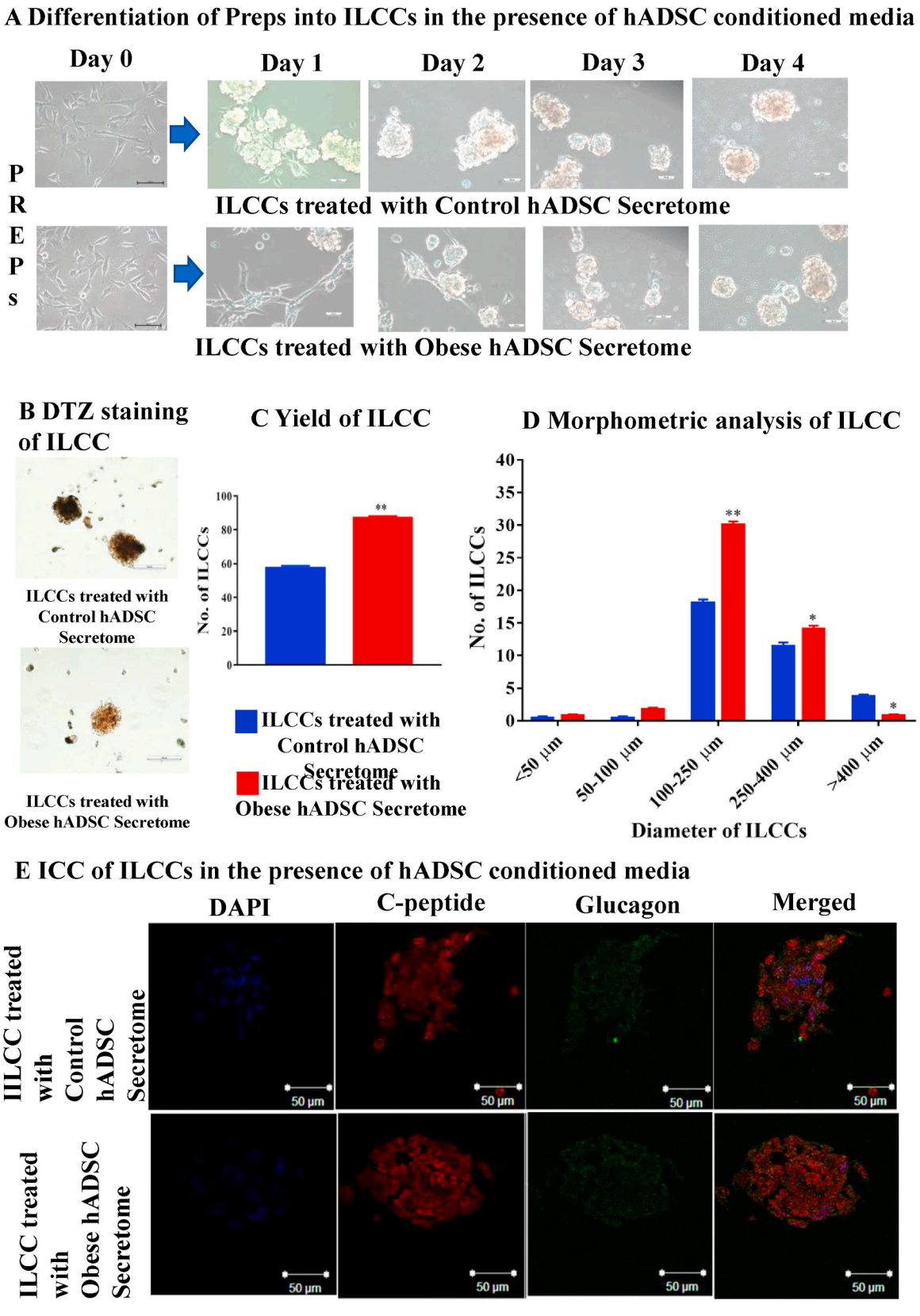

Fig. 2. Functional analysis of ILCCs in the presence of hADSC conditioned media ILCCs were differentiated from PREPs in presence of control and obese secretomes of hADSC. (A) Representative images of temporal differentiation of ILCC from day 0 to day 4 with control and obese hADSC secretomes. Functional parameters like (B) DTZ staining (C) Yield (D) Morphometric analysis (magnification 20×) and (E) Immunocytochemistry of C-peptide and Glucagon were done (magnification 63X).

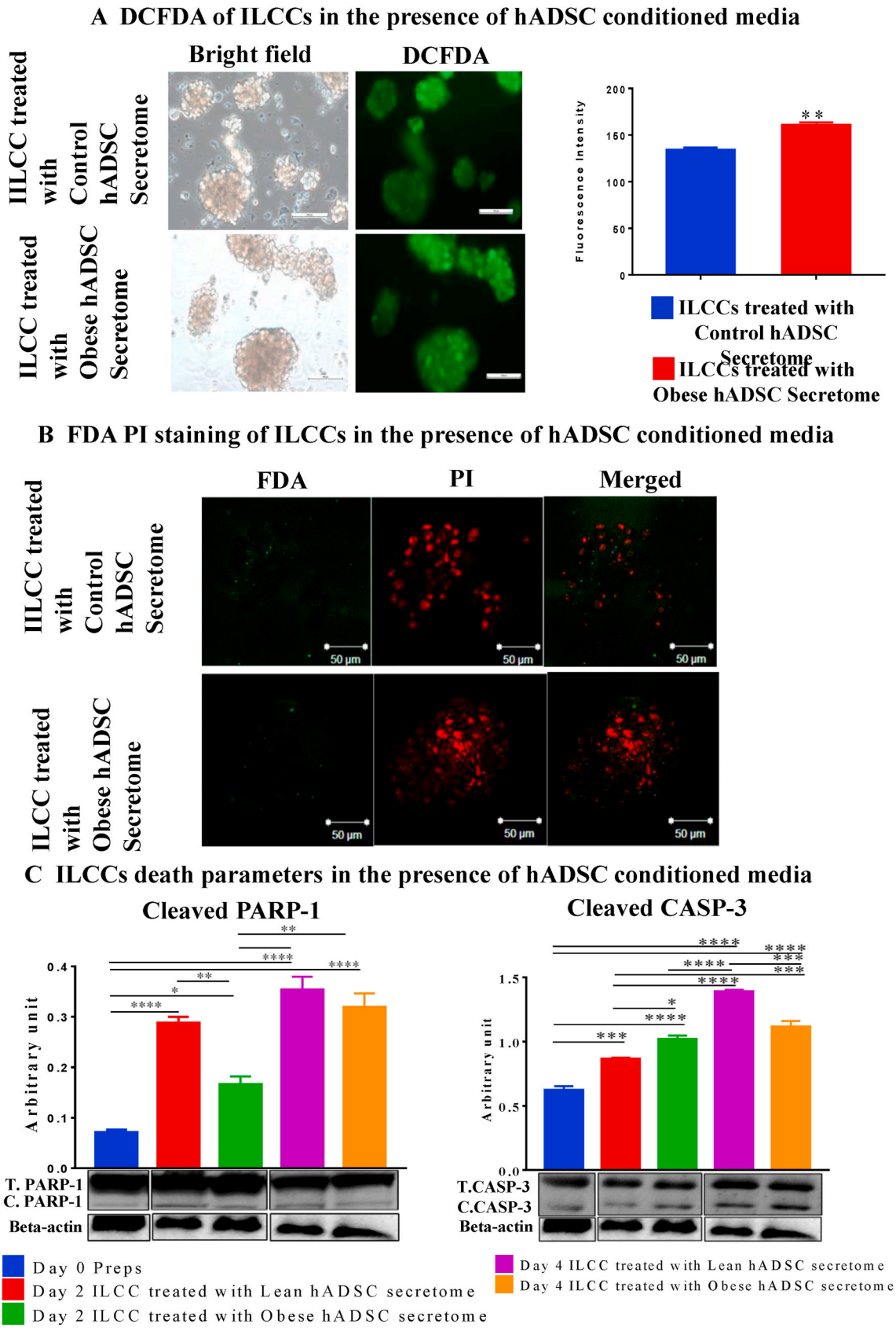

Fig. 3. Islet survival and integrity parameter of ILCCs in the presence of hADSC conditioned media ILCC were differentiated from day 0 to day 4 under the influence of control/lean and obese secretomes of hADSC. Various parameters like (A) ROS measurement by DCFDA analysis (B) Islet survival parameter by FDA PI staining and (C) PARP-1 and CASP-3 expression by western blotting were done to check islet cell death. Densitometric analysis was done normalized to Beta-actin and expressed as arbitrary unit± S.E.M. N = 3, Significance is expressed as p-value *<0.05, ** <0.01, *** <0.001, **** <0.0001.

differentiation at various stages (Fig. 4A). A series of a cascade of temporal transcriptional regulation propels pancreatic progenitors towards their inclination for islet differentiation. Stepwise differentiation of islet places FOXA2 or HNF–3B as a pioneer factor. So, we analyzed the expression of FOXA2 or HNF–3B which plays a role in the development and is an important upregulator of PDX-1 which is redundant for pancreatic development [21]. Expression increased on day 4 compared to day 2 in control and decreased expression was found on day 4 compared to day 2 in obese. The expression gradually increased from day 0 to day 4 through day 2 under the effect of both the secretome propelling the PREPs towards differentiation. Increased expression is observed at day 2 but eventually decreases on day 4 in obese compared to control. We then analyzed the expression of PDX-1 protein which is necessary for initiation of differentiation and it was found unchanged right from day 0 to day 4 in both groups except for upregulated expression in day 4 ILCCs compared to all the other groups. Next, we analyzed the master regulator NGN-3 [22] which is a redundant marker of progenitor state. The expression was considerably high on day 0.

Islet needs to determine and maintain its identity. Few of the transcriptional factors are important exclusively for maintaining the endocrine identity. For NeuroD [23] exclusive expression was observed on Day 4 in both control and obese groups compared to day 0 and day 2. Upregulation was observed in obese group compared to control on day 4. On day 1 Maf-A [24] expression was increased as compared to other groups. On day 2 and day 4 expression was increased in obese compared to control. Day 2 and day 4 control is upregulated than day 2 control. Day 4 obese is upregulated than day 4 control and obese. Finally, GLUT-2 [25] which is the most important glucose transporter was downregulated in day 4 obese islet (Fig. 4B).

This result further strengthens the fact that islet differentiation was indeed altered by obese secretome in a detrimental manner lingering and delaying maturation and differentiation and thus yielding metabolically dysfunctional and sub-optimal islet like cell clusters. These results give indisputable evidence for differential regulation of islet differentiation under normal and pathological conditions.

4. Discussion

The present study provides considerable insight into the effects of hADSCs-CM on islet differentiation through biological regulation. hADSC-CM provides a beneficial effect on islet functionality. hADSC cell free derivatives provide a range of therapeutic applications in regeneration and repair from wound healing to skin aging, to scar regeneration and neuroprotection. The enormous clinical applications make hADSC-CM a reliable therapeutic option [26]. Direct or indirect crosstalk between the pancreatic islet and hADSC has also given insight into the functional relationship between the two. Bhang et al. studied both direct and indirect effects of hADSC on rat islet viability and functionality and found that apoptotic activity of islet decreased with increased insulin secretion when subjected to hADSCs-conditioned medium [27]. But the present study not only highlights islet functionality but also the important aspect of hADSC in islet differentiation.

The metabolically harsh environment detrimentally affects stem cells. The secretome of the cell gets altered in cases of obesity along with the characteristic of hADSCs [9]. Considerable insight is followed in the recent study to explore the effects of hADSC CM under normal and pathological conditions which ultimately affect islet biology.

It is very critical to note that the islet pathophysiology was detrimentally affected by secretome from hADSC from obese. These discrepancies were further supported by the differential expression of proteins found in the secreted milieu of the hADSCs. Most of the proteins were classified under different protein classes which gave an overview of the generalization of broad categories of the proteins. This widens our knowledge of secretome alterations under pathological conditions. We have later considerably correlated the protein expression with subsequent effect on islet differentiation and functionality.

The Cluster formation with more participation of PREPs, maximum cell aggregation and zone of activation in ILCCs under the effect of control hADSC secretome has been observed as contrast to obese. This points towards the confirmation of the maturation of ILCCs with higher expression of C peptide and glucagon, an unprecedented marker of islet functionality [28] Various reports have shown that *in vitro* exposure of islets to hADSC secretome enhances islet functionality and viability [6]. Population of ILCCs was heterogenous with higher yield under obese hADSC secretome with mostly ILCCs falling under 100–200 μm range [29] These results reinforces the fact of islet cell dysfunctionality prevails under undesirable effect of obese hADSC secretome.

Islet with obese secretome had more fluorescence staining from DCFDA. In a glucotoxic environment accumulation of ROS is observed in islet since there is an inherent lack of scavenging mechanism [30]. Fluorescein diacetate/propidium iodide (FDA/PI), demonstrates reduced differential staining of intact islets [31]. Therapeutic effects of hADSC are impaired in metabolically compromised conditions [32]. Caspase 3, an apoptotic executor [19] and PARP-1, DNA repair enzyme [20] which gets activated and directs cells towards apoptosis were found upregulated in islet with obese secretome. Yamada et al.demonstrated enhanced functional and survival of porcine islets with indirect coculture with hADSC [33]. This concurs well with the decreased islet cell viability which emphasizes the fact of reaching limited functional and vital potential by the islets.

The remarkable correlation from this study is between the normal and pathological condition and the effect it has on islet differentiation. The observation to emerge from the data is the developmental effect on an islet which is unexplored in other studies.

HNF–3B plays a very important role in controlling the morphogenesis of the pancreas. Lee et al. demonstrated that lack of HNF–3B in beta cells makes the mice severely hypoglycemic and has dysregulated insulin secretion [34]. In our study, it is demonstrated that obese secretome causes a decrease in expression of HNF–3B which causes dysregulated maturity of the newly formed islet.

PDX-1 is an important differentiation factor that is redundant in maintaining beta cell identity along with beta cell differentiation and islet maturation [35]. PDX-1 was found overexpressed in day 4 islet under obese secretome. Overexpression of PDX-1 enhanced maturation but fail to respond to increased glucose. HNF–3B is also responsible for PDX-1 transcription which itself is differentially expressed under effect of secretome [34]. It also governs pancreas morphogenesis and generation of NGN-3+endocrine progenitors which is differentially expressed under secretome effect. It is reported that abrogated formation of islets results with disrupted expression of NGN-3 early in mouse pancreas development since NGN-3 is important for endocrine cell development of the islets [36].

NeuroD is overexpressed in obese secretome compared to control. NeuroD maintains the functional identity of beta cells and is a requisite for immature to mature transition during the beta cell developmental stage [23].Maf-A was overexpressed in islets under obese secretome. He et al. reported inhibition of differentiation during enforced expression of Maf-A in endocrine progenitors [24]. GLUT-2 was less expressed in islets when subjected to obese secretome which is essential for glucose sensing and insulin secretion [25].

The secretome comprised of different proteins which were differentially expressed and regulated in control and obese hADSC secretome. The majority of keratin proteins were expressed in control hADSC secretome and lesser expressed in obese hADSC secretome. Blessing et al. reported a reduced number of insulin vesicles in pancreatic beta cells by transgenic targeting of K1 or K10 which ultimately leads to diabetes [37]. The absence of keratins leads to dysfunctional glucose uptake as keratin dynamics affect glucose transporters [38].

We found an abundance of vimentin in control secretome and lesser in obese. Krivova et al. speculated vimentin as an early endocrine pancreas disorder marker. Hypertrophy and hyperplasia of islet was associated with the presence of vimentin and glycogen or insulin

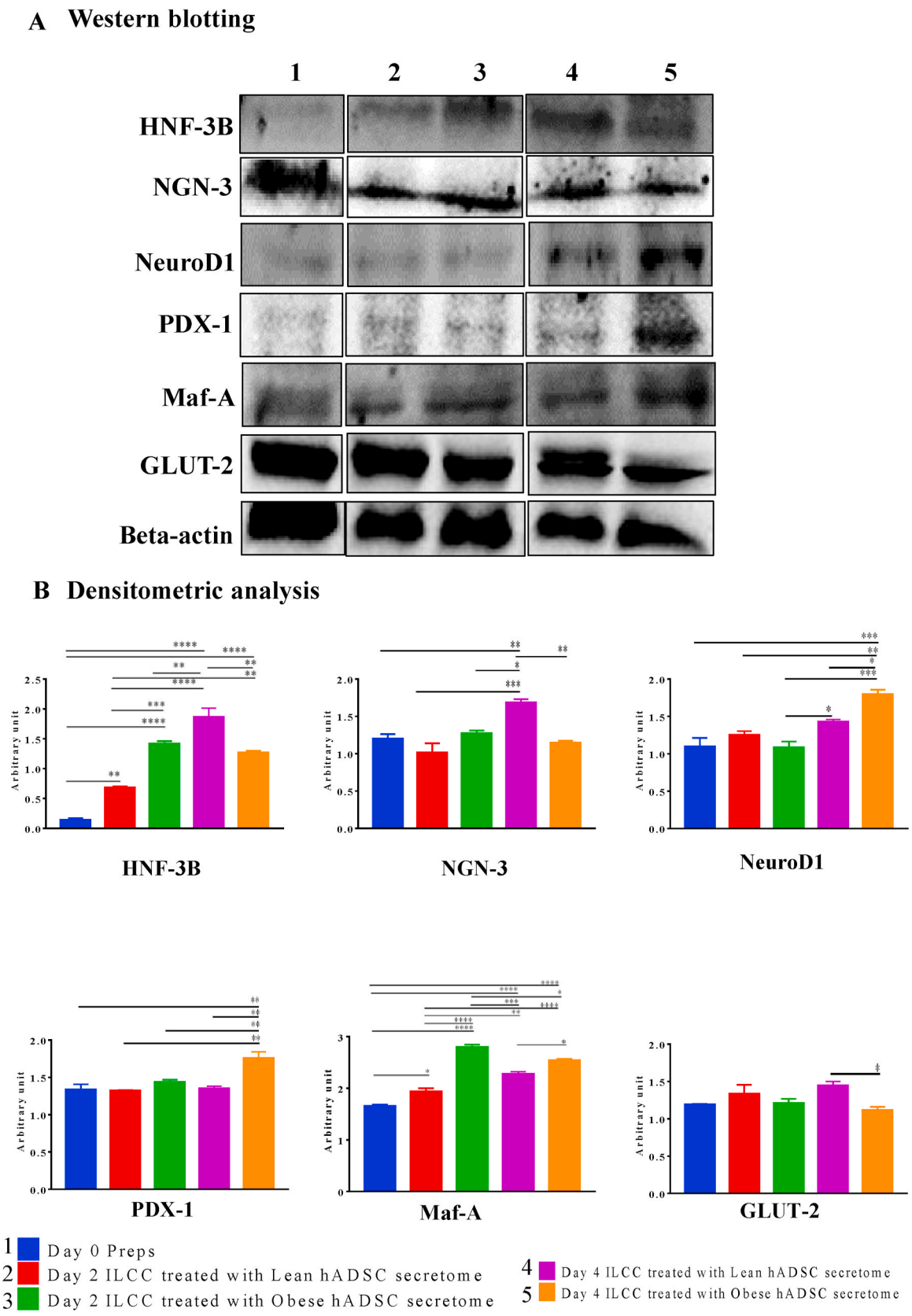

Fig. 4. Temporal analysis of proteins involved in islet differentiation Differentiation of ILCCs took place from day 0 to day 4 from PREPs with secretomes of control/lean and obese hADSC. (A) Western blotting of HNF–3B,NGN-3,NeuroD1,PDX-1,Maf-A and GLUT-2. (B) Densitometric analysis was done normalized to Beta-actin and expressed as arbitrary unit± S.E.M. N = 3, Significance is expressed as p-value *<0.05, ** <0.01, *** <0.001, **** <0.0001.

positive cells in islets of macrosomic infants from both diabetic and nondiabetic mothers [39]. Expression of vimentin was associated with loss of islet cell identity since it indicated plasticity and dedifferentiation. Moreover, also associated with reduced expression of Nkx6.1 and PDX-1 though they were not apoptotic. All these lead to beta cell dysfunction leading to metabolic disorder [40]. Vimentin was less expressed in control secretome and Wang et al. demonstrated that some nestin positive cells are heterogeneous which may lack co-expression with vimentin [41].

Myosin was abundantly present in obese secretome compared to control secretome. Intracellular movement of hormone granules in pancreatic islet is redundant for regulatory secretion of hormones which is controlled by Ca2+/CaM-dependent phosphorylation of Myosine light chain [42]. This phosphorylation mechanism also facilitates lower Ca2+ requirements than needed for the release of secretory granules [43]. Most of the ATP synthase subunits were not found in control secretome but abundantly present in obese. Li et al. observed decreased expression of ATP synthase β-subunit (ATPsyn-β) in pancreas islets of rat model of PCOS with T2DM which when upregulated by transfection also improves ATP content in islet [44]. ATP synthase subunits were exclusively expressed in obese secretome. Increased expression is observed to be a compensatory response to enhanced metabolism under hyperglycemic condition [45].

Dermicidin, a stress induced protein, was abundant in control secretome than obese. DCN2 is found in the serum of diabetic patients which is associated with disease pathogenesis. It increases insulin resistance and is ultimately responsible for lowering GLUT4 expression [46].

Transthyretin was abundant in obese secretome which is an amyloid fibril protein. Transthyretin reactive cells including beta cells are found in islets from type-2 diabetic patients since diabetes alters the expression of transthyretin [47].

Fructose biphosphate aldolase was exclusively expressed in obese secretome. Gerst et al. suggested increased expression of ALDOB resulting from hyperglycemia, is associated with lower insulin secretion in human beta cells [48].

In addition to the proteins, several studies have also found bioactives, cytokines, growth factors, circulating DNA, RNA and extra cellular vesicles etc. to be a part of the secreted milieu, so there is a possibility that such small molecules could have contributed to some extent to the observed outcomes apart from the proteins discussed in the current study [26,49–51].

The impact of the conventional secretome therapeutics have been recently replaced by the increased attention received by extracellular vesicles like exosomes and microvesicles which presents as current limitation of the present study. Owing to their emerging role for biomarkers, future consideration is highly anticipated.Thus the present study gives an account of indirect crosstalk of hADSC and islets which widens the understanding of secretome therapeutics and investigates islet functionality and differentiation under pathological effect.

5. Conclusion

Cellular therapy is the best approach for regenerative medicine. However, the cell transplantation techniques are invasive and subjected to multilevel hurdles. So an alternative secretome administration for treating pancreatic islet etiopathology in diabetes would be an effective therapeutic approach. This study will add to the knowledge on effect of altered hADSC secretome on islet functionality and differentiation which will provide greater understanding of cross talk between hADSC from adipose tissue and pancreatic islets. Thus, the cell free secretome therapy can be approached therapeutically and ease the burden of diabetes in healthcare management.

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Authors contribution

GB: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft preparation. **MV:** Supervision, Methodology, Validation, Reviewing and Editing. **AS:** Supervision, Methodology. **KR:** Methodology (*in vitro* hADSC isolation). **AK:** Methodology, Investigation, Data curation. **SG:** Conceptualization, Visualization, and Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Review Article

A PERSPECTIVE ON REGENERATIVE POTENTIALS OF HERBS FOR DIABETES THERAPEUTICS

Abhay Srivastava, Mitul Vakani, Gurprit Bhardwaj, Sarita Gupta*

Molecular Endocrinology and Stem Cell Research Lab, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

**Corresponding author: sglmescrl@gmail.com*

ABSTRACT

Diabetes Mellitus, one of the leading metabolic disorders, has become a worldwide pandemic and socioeconomic challenge where one in eleven adults are suffering from this condition. India is among the leading country that due to its populace's changing lifestyle and genetic predisposition is harboring nearly 77 million diabetic patients. Current hypoglycemic drugs in the market that are used in the treatment of diabetes can merely manage the condition and have side effects. Hence, improved and effective strategies harboring herbal medicinal plants and bioactive drugs are needed in the paradigm of diabetes therapeutics. Various neutraceutical plants/bioactive focusing on anti-hyperglycemic, increased insulin secretion, pancreatic/beta cell regenerative properties, islet-neogenic properties, are stated. The positive antidiabetic effect and nutritional value of *Enicostemma littorale* have been extensively explored. Pancreatic/beta cell regenerative properties combined with the current stem cell/ regenerative technology demonstrating differentiation potential into insulin producing cells can thus become an attractive strategy for the therapeutic intervention. The traditional nutraceutical/ herbal medicines can provide an effective alternative to the synthetic side effects of the existing diabetes drugs which will not only improve our existing knowledge but will provide a novel effective clinically acceptable diabetic cure.

Keywords: Herbal therapy, Diabetes Mellitus, Stem cells, Bio-active molecules.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that affects a large number of people. Patients suffering from diabetes continue to increase worldwide. The prevalence of both Type 1 and Type 2 diabetes mellitus is increasing throughout the world along with the ensuing morbidity and early mortality because of premature microvascular and macrovascular disease. The international diabetes federation in the year 2019 stated that around 463 million adults were suffering from diabetes. Statistically, India is severely afflicted by DM, there were over 77 million cases of diabetes in India in 2019 [1]. Diabetes is a complex metabolic disorder associated with hyperglycemia, hyperlipidemia, and oxidative stress either due to decreased insulin secretion (type 1) or lack of insulin response (type 2). In type 1 diabetes, there is the self-destruction of insulin producing pancreatic β-cells whereas in type 2, long term insulin resistance gets transformed into type 1 with loss of β-cells, which creates a chaotic state for β-cells [2,3]. There are different approaches for the treatment of diabetes; first and foremost is insulin itself, apart from this, many other drugs are in the market which target different levels such as Sulfonylurea, which efficiently releases insulin from β-cells by blocking the ATP-sensitive potassium channels. Meglitinides like repaglinide and nateglinide are other important insulin secretagogues [4, 5]. Biguanides, decrease insulin resistance, a thiazolidinedione, increase insulin sensitivity; alpha-glucosidase inhibitors like acarbose, decreases glucose absorption from the intestine, and Sodium-Glucose Cotransporters inhibitors (SGLT) cause increased glucosuria thereby decreasing hyperglycemia. Clinical trials indicate that, in some instances, control of blood glucose can be restored by transplantation of cadaveric derived and *in vitro* newly differentiated islets.

2. TRADITIONAL NUTRACEUTICAL/HERBAL PLANTS WITH ANTIDIABETIC ACTIVITY

In recent years, the popularity of complementary medicine such as traditional herbal therapy, described

by ayurvedic and indigenous systems of medicine in India has increased. Herbal preparations/agents are preferred, antidiabetic agents. Table 1 demonstrates traditional Nutraceuticals/herbal plants with anti-diabetic activity.

Table 1: Summarizing traditional Nutraceuticals/herbal plants with antidiabetic activity

Anti-Hyperglycaemic effect	Increased Insulin secretion
Azadirachta indica [6]	*Allium sativum* and *Allium cepa* [7]
Aegle marmelos [8]	*Aloe vera* [9]
Curcuma longa Linn [10]	*Acacia Arabica* [11]
Catharanthus roseus [12]	*Gymnema sylvestre* [13]
Emblica officinalis Gaertn [14]	*Ocimum sanctum* [15]
Ginseng species [16,17,18]	Insulin-mimetic property *Coccinia indica* [19]
Momordica charantia [20,21]	
Mangifera indica L [22]	
Pterocarpus marsupium [23]	
Silibummarianum [24,25]	
Trigonella foenum-graecum [26,27]	

3. COALESCING MEDICINAL PLANT/BIO-ACTIVE MOLECULES WITH REGENERATIVE MEDICINE FOR DIABETES TREATMENT

Currently, commercially available drugs manage the diabetic condition but portray health risks hence medicinal plants or natural bioactive molecules derived from herbs with pancreatic/islet regenerative properties with regards to differentiation potential from various stem/progenitors into insulin producing islets like clusters with minimal to no side effects gives an improved edge to the regenerative medicines.

4. STEM CELLS IN ISLET REPLACEMENT THERAPY

Stimulation of endogenous tissue regeneration is an important avenue that is being examined for β-cell replacement therapy. The capacity to repair tissue following an insult has been demonstrated in many tissues, including the skin, liver, and heart [28]. This mechanistic tissue regeneration has also been demonstrated in many animal models of pancreas injury such as partial duct ligation and partial pancreatectomy or by β-cell specific injury with alloxan and Streptozotocin [29]. These studies have attempted to identify the source of new β-cells following regeneration as a means to generate an increased supply of β-cells. There are three main sources of new β-cells: replication, islet ductal neogenesis, and stem/ progenitor cell differentiation.

The islet transplantation therapy for type 1 diabetes patients pioneered in 2000 by the Edmonton group [30] demonstrated its potential with remarkable freedom from exogenous insulin-dependence for the majority of patients for up to 1-2 years. Although there are limitations of this approach because more than 50% of the patients were back on insulin in five years, some continuing function of residual grafts is required [31]. Islet therapy is a promising approach. Stem cells both embryonic and adult, along with induced pluripotent stem cells (iPSCs) opens new vistas in pancreatic regenerative or islet replacement therapy. These cell-based therapies may eventually provide new rays of hope for curative treatment for diabetes. The availability of this treatment option is limited due to (i) the death of cadaveric islet donors [32], (ii) generation of new islets from stem cell pool, and (iii) availability of islet neogenic or differentiating agents [33]. However, hurdles of islet transplantation can be significantly improved. Medical practitioners had started using islet transplantation therapy to treat diabetes in the year 2000 [34]. Various methods have been developed to create *in vitro* large quantities of glucose-responsive functional islets from the pancreatic stem or precursor cells under well-defined stimulus conditions [35, 36]. Another more famously used technique is the use of human embryonic stem cells by "Novocell protocol". This when used in a defined sequence, and with specific differentiating agents helps to direct the stem cells finally towards hormone expressing group of endocrine cells which represent "islets" [37]. It is ideal that islets created *in vitro* have all the cells present in natural islets, but at least they must have majority of insulin-releasing

β-cells mixed with a small fraction of α-cells so that the "new islet cluster" can independently maintain glucose homeostasis by tightly regulating the release of insulin in response to glucose. Various differentiating agents for this rationale are now being rummaged around for. Biological growth factor like Keratinocyte growth factor (KGF), Fibroblast growth factor (FGF), Glucagon-like peptide-1(GLP-1) and Betacellulin [38-40] and chemical agents like Nicotinamide, Activin-A, Exendin-4 [41-43] are conveniently used by researchers. But the yield of islets after differentiation is not sufficient to overcome the verge of demand and the high cost of therapy. Hence scientists are now shifting towards the use of dramatic medicinal properties of herbal plants that may possess islet neogenic activity.

5. MEDICINAL PLANTS IN PANCREATIC REGENERATION AND ISLET NEOGENESIS:

5.1. Citrullus colocynthis

According to the findings of one of the studies, the application of 125 mg *C. colocynthis* once per day for 2 months can lead to a considerable decrease in the mean levels of HbA1c and FBS among patients with type II diabetes without any side effects [44]. *C. colocynthis* aqueous seed extract stabilized animal body weight and ameliorated hyperglycemia in a dose- and time-dependent manner which was attributable to regenerative effect on β cells and intra-islet vasculature. It increased the islet diameter and β cell count [45].

5.2. Tinospora cordifolia

Authors have reported that treatment with *T. cordifolia* stem powder caused a reversal in the level of fasting blood sugar by 9 % in Type 2 diabetic patients compared to control human controls [46]. The novel polysaccharide from *T.cordifolia* possesses hypolipidemic and hypoglycemic properties. It also has glucose oxidizing and β cell regenerative properties. Regeneration of β cells in the pancreatic sections was found in the histological studies of Streptozotocin-induced Diabetic Wistar Rats [47].

5.3. Moringa oleifera

Kumari *et al*. demonstrated the treatment of type 2 diabetic subjects with 8 g of powdered *M. oleifera* leaf in a tablet form per day for 40 days. A total of 46 subjects were involved in the study. At the end of the study, fasting blood glucose and postprandial blood glucose were 28% and 26% lower, respectively, in the treated subjects [48]. Aqueous extract of *M. oleifera* leaves possesses potent hypoglycemic effects through the normalization of elevated hepatic pyruvate carboxylase enzyme and regeneration of damaged hepatocytes and pancreatic cells via its antioxidant properties. Moreover, it restored the normal histological structure of the liver and pancreas damaged by alloxan in diabetic rats [49].

5.4. Ephedra sinica Stapf, and Ephedra distachya

Glycans and ephedrans A, B, C, D, and E, isolated from *Ephedra distachya* herbs have been confirmed to have anti-hyperglycemic activity in alloxan-induced diabetic mice by regenerating atrophied endocrine pancreas and restoring insulin secretion [50].

5.5. Oreocnide integrifolia:

Here, the study examined the potential of the flavonoid-rich fraction of *Oreocnide integrifolia* in pancreatic regeneration of 70% pancreatectomized BALB/c mice. Although they explained that the ductal progenitors were responsible for the pancreatic regeneration, they did not comment on either the active principle responsible for the flavonoid fraction or its mechanism of action for the same [51]. Figure 1 depicts the islet neogenic activity of various herbal plants and table 2 describes medicinal plants with pancreatic/ β cell regenerating ability.

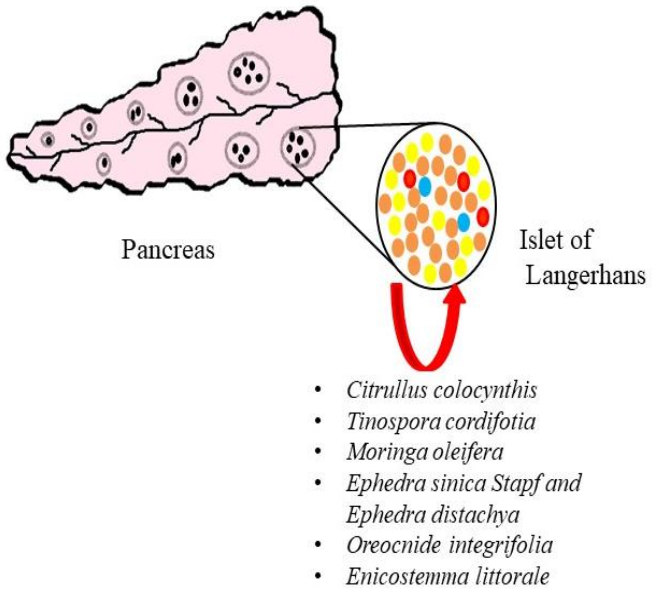

Fig. 1: Islet neogenic activity of various herbal plants

Table 2: Plants with pancreatic/ β cell regenerating ability

Plants with pancreatic/β cell regenerating ability	Mode of action	Reference
Citrullus colocynthis	Regenerative effect on β cells and intra-islet vasculature	[45]
Tinospora cordifolia	β cell regenerative properties	[47]
Moringa oleifera	Regeneration of damaged pancreatic cells via its antioxidant properties	[49]
Ephedra sinica Stapf, and Ephedra distachya:	Acts by regenerating atrophied endocrine pancreas and restoring insulin secretion	[50]
Oreocnide integrifolia	Pancreatic regeneration of 70% pancreatectomized BALB/c mice.	[51]

6. *ENICOSTEMMA LITTORALE:* ANTI DIABETIC EFFECTS AND A NEW TARGET FOR ISLET NEOGENESIS

Enicostemma littorale (Gentianaceae) belonging to the family Gentianaceae is a glabrous perennial herb. It grows throughout India up to 1.5 feet high and more frequently near the sea. It is called Chota-kirayat or Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal, and Vellarugu or Vallari in Tamil.

As for the high nutritional value of EL, 2 g of EL fresh leaves is daily recommended in diabetes [52]. A 100 g of fresh EL contains 140 kcal energy with 26.5g of carbohydrate, 7g of protein, 0.7g of fat, 8.4 g of minerals, 1.641 mg of calcium, 49.9 mg of iron, 81mg of phosphorous, and 4.2 g of fiber as per the nutritional analysis report of Indian Council of Medical Research [53].

Traditionally aqueous extract and dried powder of this herb have been used for the treatment of malaria and diabetes, however was not evaluated scientifically till the year 2000. Taking lead from the reverse pharmacology approach, studies with aqueous extract of this plant were initiated [54, 55]. Alloxan-induced diabetic rats showed increase in serum insulin levels owing to hypoglycemic and antidiabetic effects of aqueous extract of *Enicostemma littorale* along with insulin secretagogue action in isolated rat pancreatic islets [54, 56, 57]. The methanolic extract imparts an insulinotropic effect, normalizes dyslipidemia, and reduces oxidative stress [54, 55]. In another study, diabetic rats when treated with plant extract for 45 days showed a reduction in blood glucose levels and also provided nephron, neuro, and cardioprotective effects [58-60].

It is also reported that the EL extract improved lipid profile at a small dose of 0.5 g/kg [61]. Our group has also suggested that the methanolic extract of *Enicostemma littorale* imparts cytoprotective and anti-apoptotic effects to the islet of Langerhans against oxidative stress [62]. Apart from animal studies, we have also reported the antidiabetic effect of EL in Non-insulin dependent diabetes mellitus (NIDDM) patients showing hypoglycemic, antioxidant, and hypolipidemic actions with aqueous extract [63]. Table 3 demonstrates *Enicostemma littorale* extracts and its anti diabetic properties

Table 3: *Enicostemma littorale* Plant extracts & anti diabetic properties

Enicostemma littorale	**Aqueous Extract** Hypoglycemic effect, insulin secretagogue action & Cardioprotective.	[54,56,57]
	Methanolic Extract The hypoglycemic, insulinotropic effect, Hypolipidemic, antioxidant as well as preventive effects of Nephrotoxicity & Neuropathy. Moreover, Methanolic extract recently demonstrated Cytoprotective and anti-apoptotic effects on the islet of Langerhans against oxidative stress.	[54,55] [58-60] [62]

7. PANCREATIC REGENERATIVE AND ISLET-NEOGENIC PROPERTIES OF HERBAL AGENTS (BIOACTIVE)

7.1. Geniposide

Geniposide significantly decreased the blood glucose, insulin as well as triglyceride levels in diabetic mice. It also promotes β cell regeneration and survival in i*n vitro* study in isolated mouse islets & mouse pancreatic cell line (MIN6). Further, mouse pancreatic islets *in vitro* as well as mouse *in vivo* study identified a role of geniposide in enhancing β-cell survival and regeneration

by mechanisms involving the activation of β-catenin/T-cell factor 7 like-2 signaling pathways [64].

7.2. Kinsenoside

Kinsenoside from *A. roxburghil* is a major component of its n-butanol fraction, exhibited glucose lowering effect in STZ rats at 15 mg/kg dose along with increased glucose tolerance. Enhanced integrity of islets of Langerhans was observed in the Kinsenoside treated rats, which indicated pancreatic β-cell regeneration. Thus, rendering Kinsenoside as a promising antidiabetic agent for therapy [65].

7.3. Silymarin

Silymarin recovers the normal morphology and endocrine function of damaged pancreatic tissue in alloxan induced diabetic rats. Moreover, the Silymarin treatment induced an increase in both Pancreas/ duodenum homeobox protein 1 (*Pdx1*) and insulin gene expression as well as β-cell proliferation in Wistar rats, partially pancreatectomized (60%) model [66]. Five randomized human clinical trials on 270 diabetic patients showed that routine silymarin administration regulates a momentous reduction in fasting blood glucose levels as well as HbA1c levels [67].

7.4. Genistein

Genistein enhances insulin secretion & inhibits pancreatic β-cell apoptosis. Genistein treatment increased β-cell proliferation in cell culture models as well as in the pancreas of Genistein-treated diabetic mouse model. The effects appear to involve cAMP/ PKA (cyclic Adenosine monophosphate/Protein kinase A) signaling. Genistein treatment was also associated with increases in intracellular cAMP, PKA activity, and active ERK1/2 (extracellular signal-regulated kinases), suggesting that the cAMP/PKA and ERK1/2 pathways are stimulated by Genistein treatment. Identical effects were observed in human islet β-cells that were exposed to Genistein, suggesting non-species-specific and human-relevant effects [68]. Randomized Human clinical trials revealed that 1-year genistein treatment significantly reduced fasting glucose and fasting insulin as well as HOMA-IR (Homeostatic model assessment (HOMA) insulin resistance (IR)) without any side effects [69].

7.5. Quercetin

Commonly found in plants (In many fruits, vegetables, leaves, and grains) enhances insulin secretion and inhibits pancreatic β cell apoptosis. Quercetin potentiated both glucose and Glibenclamide-induced insulin secretion in the insulin-secreting cell line INS-1 and rat isolated pancreas. The ERK1/2 signaling pathway played a crucial role in the potentiation of glucose-induced insulin secretion by quercetin. Also, quercetin protected β-cell function and viability against oxidative damage induced by H_2O_2 and induced major phosphorylation of ERK1/2 [70].

7.6. Berberine

Berberine is isolated from *Rhizoma coptidis*. It enhances insulin secretion and lowers hyperglycemia. It stimulated pancreatic β cell regeneration in type 2 diabetic animals [71]. Moreover, the pilot human clinical trials study on 36 types 2 diabetic patients for 3-month treatment of berberine revealed that significant decreases in hemoglobin A1c, fasting blood glucose, postprandial blood glucose [72].

7.7. Nymphayol

Nymphayol enhances insulin secretion. Nymphayol isolated from Nymphaeastellata (Wild.) flowers demonstrated Partial regeneration of β cells [73].

7.8. Ginsenoside

Ginsenoside Rg3 (Rg3), an active ingredient of ginseng saponins, has been reported to enhance insulin secretion stimulating anti-apoptotic activities in pancreatic beta cells. Ginsenoside administration might be a prospective management approach to enhance- islet function and ameliorate early inflammation after islet transplantation. Also, anti-obesity effects were observed in diabetic patients and diabetic animal models [74].

7.9. Conophylline

Conophylline is a vinca alkaloid. It is extracted from the tropical plant *Ervatamia microphylla*. Conophylline addition to pancreatic rudiments in culture as well as to AR42J cells (a model of pancreatic progenitors) increased the number of insulin, and *Pdx-1*-positive cells. Treatment with Conophylline of neonatal Streptozotocin diabetic rats increased as well the number of insulin-positive clusters budding from ductal structures. This resulted in glucose normalization and improved glucose tolerance for 2 months. The data supports the role of Conophylline in β-cell regeneration, which has also been described in the same animal model treated with glucagon-like peptide and Exendin-4. Moreover, the same group also previously

demonstrated that the number of islet-like cell clusters and pancreatic duodenal homeobox-1-positive ductal cells were greater in 2-month conophylline-treated rats. These results propose that conophylline induces differentiation of pancreatic progenitors' cells and increases the formation of Pancreatic β-cells. [75]. This compound acts as well in the *Shh* pathway, mimicking the effect of Activin-A. Besides, Activin-A, when bound to its receptor, can activate p38 mitogen-activated protein kinase inducing the expression of neurogenin3. Conophylline acting as a ligand for Activin-A was later proved by performing differentiation in presence of Activin-A antagonist. The antagonist blocked islet generation when incubated with Conophylline confirming the mode of differentiation [76]. This was the first evidence that provided some clue that herbal compounds possess islet neogenic property, thus playing a crucial role in islet differentiation.

7.10. Resveratrol

Differentiation protocols incorporating Resveratrol (RSV) treatment yielded numerous insulin-positive cells, induced significantly higher PDX1 expression, and were able to transiently normalize glycemia when transplanted in streptozotocin (STZ) induced diabetic mice thus promoting its survival [77]. A Human clinical study showed that daily resveratrol treatment for 3 months significantly decreased HbA1c levels in 28 Type-II diabetic patients [78].

7.11. Curcumin

Several studies highlight curcumin's benefit as a hypoglycemic agent as enhances insulin secretion. Researcher suggests pancreatic islets regeneration in diabetic rats when treated with Curcumin for forty days (Long term study). Anti-inflammatory and antioxidant effects of curcumin create a favorable environment to promote islet neogenesis [79, 80]. A 9-month curcumin clinical trial on humans demonstrated that Curcumin has a positive effect on the prediabetic population. Besides, the curcumin treatment appeared to recover the overall function of pancreatic β-cells, with very lesser adverse effects [81]. Figure 2 demonstrates the islet neogenic activity of various plant bioactive.

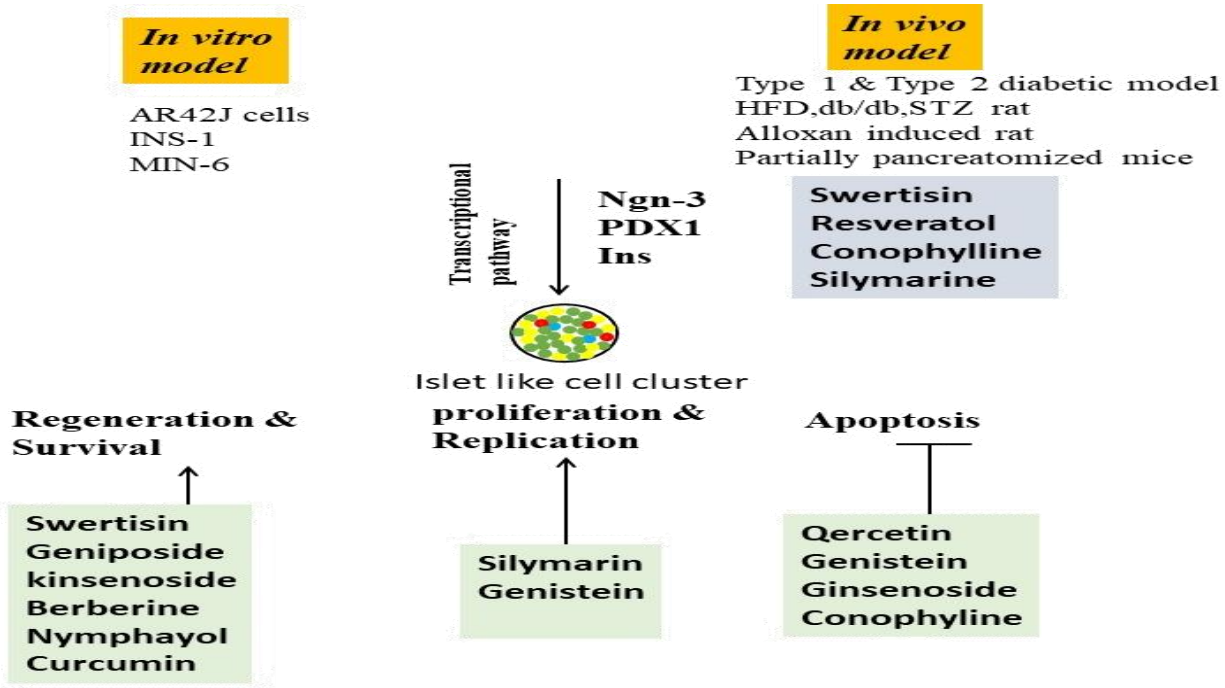

Fig. 2: Islet neogenic activity of various plant bioactives

8. ANTIDIABETIC ACTIVITY OF ISOLATED COMPOUNDS FROM *ENICOSTEMMA LITTORALE*:

Many compounds have been isolated and reported from *Enicostemma littorale*. Five alkaloids, two sterols, and volatile oil have been reported by Natarajan and Prasad [82]. Seven flavonoids including swertiamarin have been reported by Ghoshal *et.al*. [83] and swertiamarin a gentiopicroside was isolated from ethyl acetate extract [84]. Further swertisin and swertiamarin were

successfully isolated from EL and proved potent insulin sensitizer and adipogenic inhibitor property of swertiamarin in NIDDM rats and hepatic steatosis models [85,86]. Swertisin has been proved as a potent inducer of islet differentiation from stem/progenitors.

8.1. Swertisin imparts Islet neogenic activity to *E. littorale:*

Recently stem/progenitor differentiation activity of *Enicostemma littorale* has also been reported where human pancreatic carcinoma cells Panc-1 and mouse embryonic fibroblast cells NIH3T3 were converted to functional insulin producing islet clusters [87]. Dadheech et al., in 2015 identified the active principle molecule Swertisin, a flavonoid that was responsible for the above islet neogenic property. Swertisin, not only gave a better yield of islets but it was also superior in terms of the amount of insulin released after a glucose challenge. Further, the islets generated using Swertisin were transplanted into Streptozotocin treated diabetic BALB/c mice, which became normoglycemic after the transplantation. Further, the molecular mechanism of Swertisin was extensively studied and was found to follow Activin A mediated MEPK-TKK pathway during islet Neogenesis. Insulin transcript levels increased owing to swertisin. It also decreased expression of Nestin, Ngn-3 (Neurogenin-3), and Pancreatic Duodenal Homeobox Gene-1 (PDX-1) in a post (PPx) partial pancreatectomised mice model [88]. Swertisin when administered into STZ diabetic mice (*in vivo*) also triggered the resident pancreatic progenitors to replenish and recover the endocrine function by increasing islet formation [89]. All these properties of Swertisin make it an ideal candidate for a novel therapeutic intervention in treating diabetes mellitus. Hence, presently islet differentiating activity of swertisin has been explored with human mesenchymal stem cells and further our efforts are in the direction of designing potent islet therapy using plant-derived compounds for effective diabetes treatment. Table 4 demonstrates Plant bioactive with pancreatic/ β cell regenerating ability. Figure 3 depicts islet neogenic activity of swertisin.

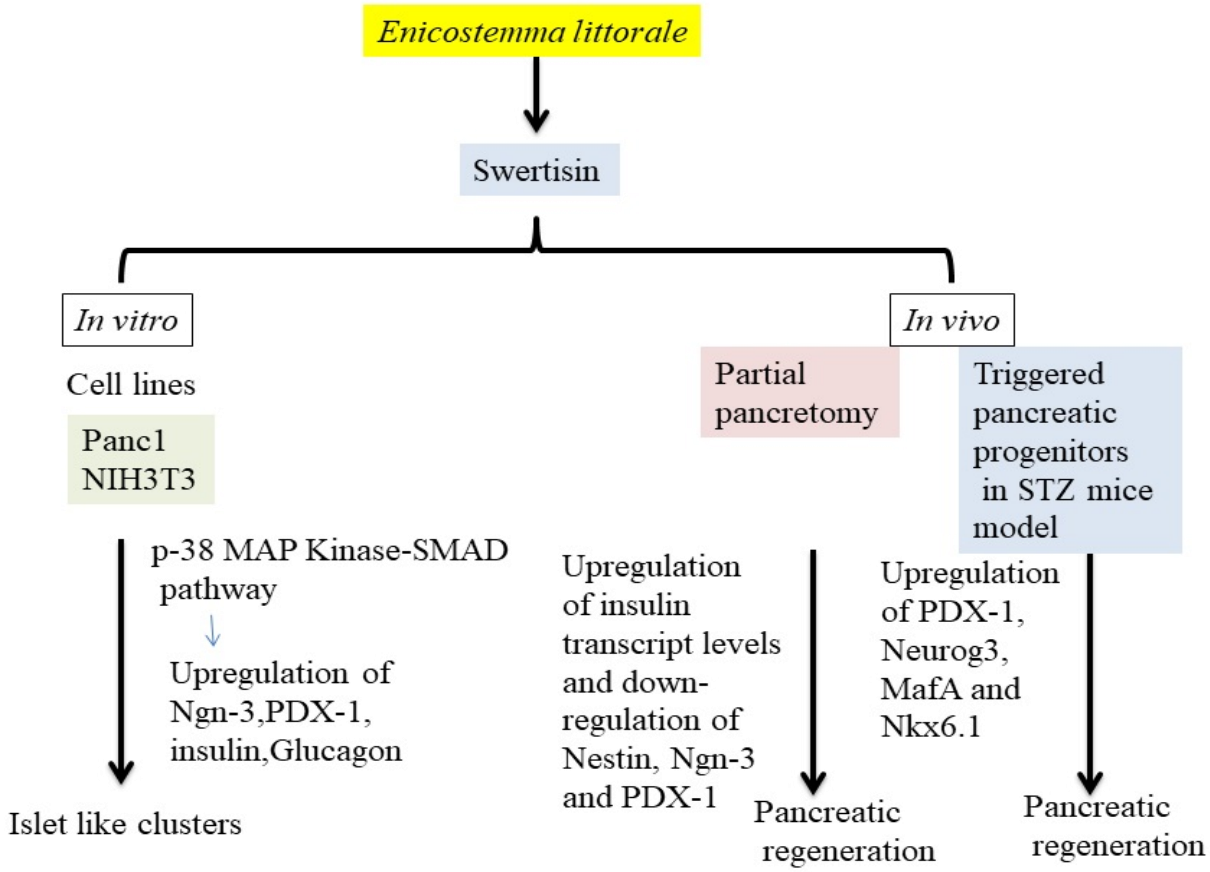

Fig. 3: Islet neogenic activity of swertisin

Table 4: List of pancreatic/ β cell regenerating bioactive agents

Sr no.	pancreatic/β cell regenerating agents	Mode of action	Reference
1.	Geniposide	Promotes β cell regeneration and survival	[64]
2.	Kinsenoside	Enhanced integrity of islets of Langerhans was observed in the Kinsenoside treated rats, which indicated pancreatic β-cell regeneration. Thus, rendering Kinsenoside as a promising antidiabetic agent for therapy	[65]
3.	Silymarin	Effect of Silymarin in *Pdx-1* expression and the proliferation of pancreatic β-cells in a pancreatectomy model.	[66]
4.	Genistein	Enhances insulin secretion & Inhibits pancreatic β cell apoptosis	[68]
5.	Quercetin	Commonly found in plants(in many fruits, vegetables, leaves, and grains) enhances insulin secretion & inhibits pancreatic β cell apoptosis	[70]
6.	Berberine	Berberine enhances insulin secretion and stimulated pancreatic β cell regeneration in Type 2 diabetic animals	[71]
7.	Nymphayol	Nymphayol enhances insulin secretion. Nymphayol isolated from *Nymphaeastellata* (Wild.) flowers demonstrated Partial regeneration of β cells.	[73]
8.	Ginsenoside	Ginsenoside Rg3 (Rg3), an active ingredient of ginseng saponins, has been reported to enhance insulin secretion stimulating anti-apoptotic activities in pancreatic beta cells.	[74]
9.	Conophyllin	Increased the number of insulin, and *Pdx-1*-positive cells *in vitro* resulted in glucose normalization and improved glucose tolerance. β-cell regeneration. Conophylline acting as a ligand for Activin-A was later proved by performing differentiation in presence of Activin-A antagonist.	[76]
10.	Resveratrol	Differentiation protocols incorporating Resveratrol (RSV) treatment yielded numerous insulin-positive cells, induced significantly higher PDX1 expression.	[77]
11.	Curcumin	Anti-inflammatory and antioxidant effects of curcumin create a favorable environment to promote islet neogenesis	[79,80]
12.	Swertisin	A flavonoid which is responsible for islet neogenic property. Swertisin, not only gave a better yield of islets but it was also superior in terms of the amount of insulin released after a glucose challenge. Further, the islets generated using Swertisin were transplanted into Streptozotocin treated diabetic BALB/c mice, which became normoglycemic after the transplantation. Further, the molecular mechanism of Swertisin was extensively studied and was found to follow Activin-A mediated MEPK-TKK pathway during islet neogenesis. Swertisin was extensively studied and was found to follow Activin-A mediated pathway during islet neogenesis. Swertisin when administered into STZ diabetic mice (*in vivo*) also triggered the resident pancreatic progenitors to replenish and recover the endocrine function by increasing islet formation.	[54,55,56, 57] [88,89]

9. CHALLENGES IN IPC DIFFERENTIATION

There are far too many inducers and various sources of stem/precursor cells used for differentiating insulin producing cells. The major challenge thus faced by the clinicians is the combination of the stem cell source and the differentiating inducer. The molecular mechanism of most of the naturally derived molecules is not well established and even if they are known, it is concerning a particular type of cellular system. Hence, the mechanistic pathway involved might differ from one cellular system to the other. Thus, it is an imperative challenge to understand the effect of these inducers not only at the pancreatic level but at the systemic level by using these molecules *in vivo*. Further cost effectiveness is another important issue for translation into clinical therapy.

10. CONCLUSION

There is enough literature and experimental evidence to suggest antidiabetic effects of various bioactive derived from herbs with few reports with regards to differentiation potential from various stem/progenitors into insulin producing islets like clusters. We have successfully demonstrated the role of bioactives from *Enicostemma littorale* having this potential and thus become the basis of a successful regenerative therapeutic strategy for treating diabetes mellitus. However, there is still a need to find more such compounds in perfect combination which can optimize the generated Islet like cluster (ILCs) in terms of yield, size, insulin production, and glucose homeostasis.

Declarations of interest

None declared

11. REFERENCES

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