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Swertisin an Anti-Diabetic Compound Facilitate Islet Neogenesis from Pancreatic Stem/Progenitor Cells *via* p-38 MAP Kinase-SMAD Pathway: An *In-Vitro* and *In-Vivo* Study

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Abstract

Transplanting islets serves best option for restoring lost beta cell mass and function. Small bio-chemical agents do have the potential to generate new islets mass, however lack of understanding about mechanistic action of these small molecules eventually restricts their use in cell-based therapies for diabetes. We recently reported "Swertisin" as a novel islet differentiation inducer, generating new beta cells mass more effectively. Henceforth, in the present study we attempted to investigate the molecular signals that Swertisin generate for promoting differentiation of pancreatic progenitors into islet cells. To begin with, both human pancreatic progenitors (PANC-1 cells) and primary cultured mouse intra-islet progenitor cells (mIPC) were used and tested for Swertisin induced islet neogenesis mechanism, by monitoring immunoblot profile of key transcription factors in time dependent manner. We observed Swertisin follow Activin-A mediated MEPK-TKK pathway involving role of p38 MAPK via activating Neurogenin-3 (Ngn-3) and Smad Proteins cascade. This MAP Kinase intervention in differentiation of cells was confirmed using strong pharmacological inhibitor of p38 MAPK (SB203580), which effectively abrogated this process. We further confirmed this mechanism in-vivo in partial pancreatectomised (PPx) mice model, where we could show Swertisin exerted potential increase in insulin transcript levels with persistent down-regulation of progenitor markers like Nestin, Ngn-3 and Pancreatic Duodenal Homeobox Gene-1 (PDX-1) expression, within three days post PPx. With detailed molecular investigations here in, we first time report the molecular mode of action of Swertisin for islet neogenesis mediated through MAP Kinase (MEPK-TKK) pathway involving Ngn-3 and Smad transcriptional regulation. These findings held importance for developing Swertisin

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as potent pharmacological drug candidate for effective and endogenous differentiation of islets in cell based therapy for diabetes.

Introduction

Islet Neogenesis refers to generation of new β-cells from progenitor cells. Insulin producing β-cells form bulk of islets (65–80%), are targeted for destruction at early stage in type I diabetes and at an advanced stage in type II diabetes. Hence, identification of novel differentiation inducer is a prime requisite for islet generation and increasing beta cell mass, which could be next generation therapeutics for diabetes. Also, there is need to understand molecular mechanism involved in β-cells differentiation using small molecule as differentiating agents. This can be exemplified by phenomenon "Ontology recapitulates phylogeny" [1]. In 2004, Melton's group conducted an elegant lineage tracing experiment to strongly argue that pre-existing terminally differentiated β-cells retain a strong proliferative capacity *in vivo* and they are the major source of new β-cells during adult life and after partial pancreatectomy in mice [2]. Their study challenged the notion that adult pluripotent stem cells could have a significant role in β-cells replenishment [3]. In parallel, Xu et al. produced equally strong evidence that new β-cells can be generated in injured pancreas of adult mouse from its endogenous (pancreatic) progenitor/stem cells [4]. Various distinct mechanisms are postulated to account for β-cells regeneration, mainly (i) trans-differentiation of exocrine cells into endocrine β-cells; (ii) emergence of new β-cells from pancreatic ductal epithelium; and (iii) replication of pre-existing β-cells and lastly (iv) stem cell differentiation from various tissue sources [5].

To expedite the process of *in-vitro* islet neogenesis from various types of progenitor cells, we need to have a better understanding of different factors and their mode of action that can influence this process. Many studies have focused on the role of small peptides, cytokines and proteins in stem cell differentiation to obtain insulin-producing cells [6]. Some of the compounds have been instrumental in islet differentiation protocols, such as Hepatocyte Growth Factor, Insulin like Growth Factor, Activin-A, Exendin-4, Glucagon Like Peptide-1, INGAP and Betacellulin etc. Most importantly, with all above experimental evidences only two molecules Activin-A and Keratinocyte growth factor (KGF) has been explored for their mechanism of action for differentiation, till date [7, 8]. Activin-A promotes islet differentiation via ACT--MEPK-TKK pathway mediated through activin (ACT-III) receptors that drive increased phosphorylation of p38 leading to activation of Ngn-3, controlling endocrine transcriptional machinery via smad proteins for islet generation [9]. Movassat et al., demonstrated that KGF promotes beta-cell regeneration by stimulating duct cell proliferation *in vivo* by directly inducing the expression of PDX1 in some ductal cells thus leading to beta-cell neogenesis. The molecular mechanism of KGF involved direct effects on duct cell proliferation, mediated by the MEK-ERK1/2 pathway, while differentiation by regulating PI3K/AKT pathway [7]. It is pertinent to note that in both the studies MAP Kinase pathway was actively involved and responsible for differentiation.

Apart from biological or chemical inducers, very few investigators have used herbal products as known differentiating agents to obtain insulin-producing cells. One such study by Kojima et al., first time reported and introduced herbal agent named "conophylline" which showed generous differentiation of pancreatic acinar AR42J cells into insulin producing cells with elevated expression of Pdx-1, Ngn3 and GLUT-2 in differentiated clusters [10]. This group has provided few superficial evidences on differentiation mechanism of conophylline;

mimicking Activin-A triggered differentiation signals *via* p38 MAP Kinase phosphorylation involving Ngn-3 up-regulation [9]. With all above reports, it is clearly highlighted that both activin-A and MAP Kinases are effectively involved in beta cell development. Therefore, for effective translational therapeutics, it becomes more important now to understand the mechanistic action of these small molecules and develop them as potential candidate for islet neogenesis.

In last few years, our lab has examined anti-diabetic, hypolipidaemic, and islet protective activity of *Enicostemma littorale* [11–13]. The plant has also been examined for its capacity in promoting islet neogenesis. Recently, we reported *in-vitro* formation of functional islet-like cell clusters containing both β and α cells from Panc-1 cells and mouse embryonic fibroblast NIH3T3 cells using small biomolecule "Swertisin" isolated from *E. littorale* [14, 15], as a potential molecule for beta cell generation. This led us to investigate the mechanistic action of this potent bioactive agent for islet cell differentiation property. We therefore attempted a systematic and time dependent study of transcriptional machinery involved in islet differentiation induced by Swertisin under both *in-vitro* and *in-vivo* conditions. Activin-A was used as control to compare the MEPK-TKK signal pathway during differentiation.

Results

In order to understand the mechanism of islet neogenesis mediated by *E. littorale* active molecule "Swertisin", we carried out time dependent monitoring of key transcriptional factors and progenitor markers implicated in islet formation using human pancreatic adenocarcinoma cells PANC-1 and mouse intra-islet progenitor cells (mIPC) along with Activin-A as control.

PANC-1 cells differentiate into ILCC with Activin-A and Swertisin

Human pancreatic progenitor is best system to understand the molecular mechanism of islet neogenesis and Panc-1 cells serves a simplest model for study, as there is no other model available for human progenitors. Panc-1 recently demonstrated to present various progenitor markers like C-kit and Stem cell factor (SCF) making Panc-1 as best suitable model for progenitor studies [16]. We conducted Panc-1 differentiation into islet like clusters with earlier described protocol [14, 15], in serum free media (SFM). Panc-1 cells were subjected to serum free medium- SFM; SFM supplemented with ITS; SFM/ITS with Activin-A and SFM/ITS with Swertisin for 8 days. We observed effective cell clustering which started on 3rd day in incubated cells, demonstrating zone of activation for endocrine reprogramming. Activin-A induced clusters turned into mature ILCCs by 8th day and showed presence of insulin, demonstrated by Dithizone (DTZ) staining on 10th day (Fig 1A). Immunostaining for insulin, c-peptide and glucagon showed mature islet formation (Fig 1A). Similar to Activin-A, Swertisin also exhibited intense crimson red to brown DTZ staining whereas those from SFM with ITS failed to stain (Fig 1B). Clusters from all three groups, when examined immunocytochemically, confirmed the presence of deep insulin staining (brick-red) in Activin-A and Swertisin clusters but weakly positive in SFM alone group (Fig 1B).

We further quantitated insulin fluorescence signal per unit cytoplasm, where we found significant increase in insulin signals both in Activin-A and Swertisin clusters upon 8 days differentiation compared to SFM control. Moreover Swertisin group showed even significant high insulin content than Activin-A group (Fig 1C and 1D). Scatter plot analysis between intensity of insulin fluorescence (alexa 488-red) and nuclear signal of DAPI (405-Blue), depicted frequency for insulin immunopositivity in differentiated clusters. When observed in SFM with ITS, scatter showed low distribution towards alexa 488 signals but more nuclear signal pixels, depicting weaker progression for beta cell differentiation fate. Comparatively, a dramatic shift

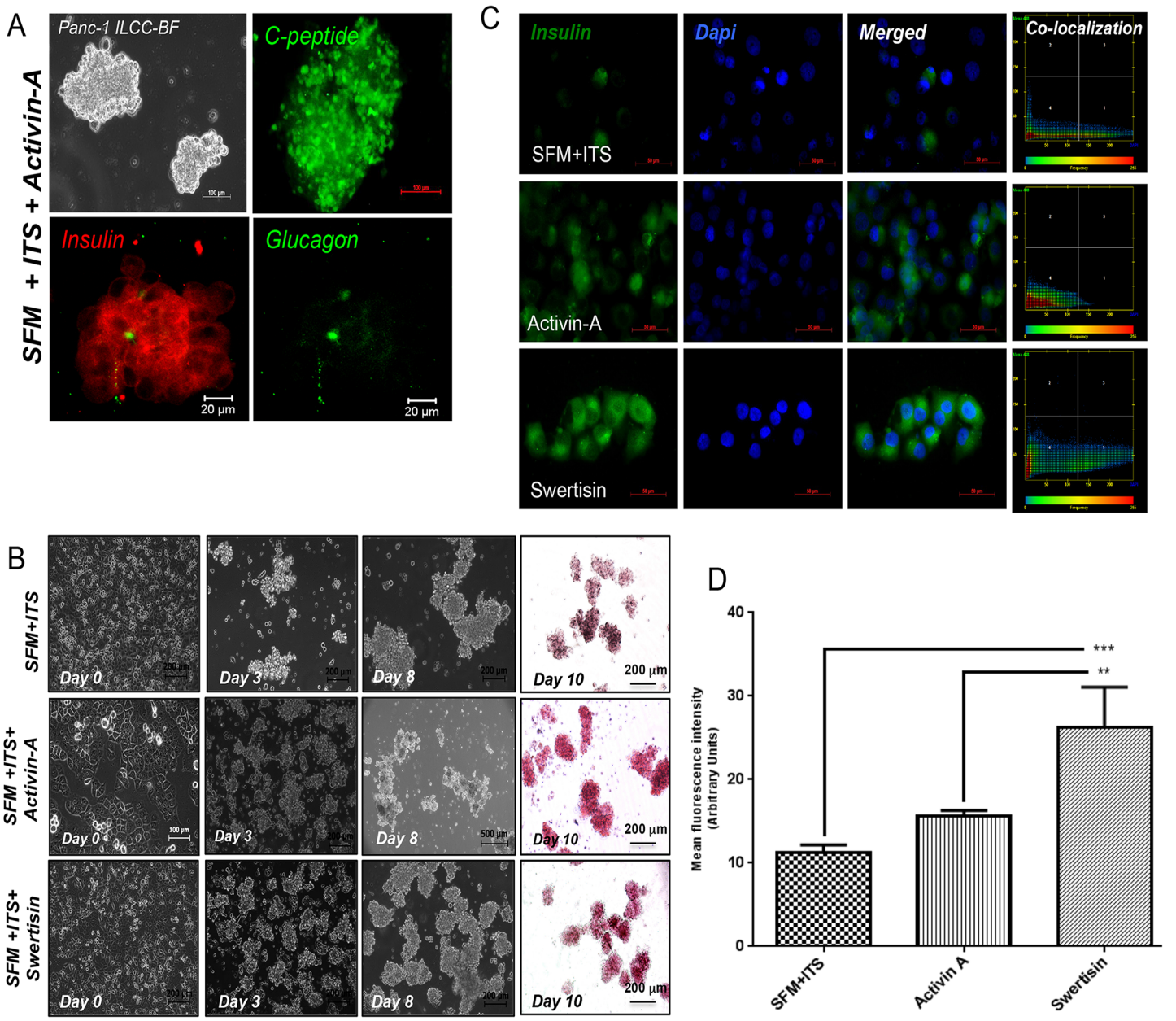

Fig 1. PANC-1 cells differentiation and characterization with Activin-A. (A) showed differentiation of Panc-1 cells subjected to differentiation using activin-A for 10 days. Figure shows bright field image of ILCC generated upon differentiation on day 10th at 10X magnification. Panels (A) showed Panc-1 ILCC immunostained on 10th day, positive for C-peptide, Insulin, and glucagon. Insulin was stained with TRITC labeled antibody showed red in color whereas c-peptide and glucagon were stained using FITC labeled antibodies showed green in color. (B) shows comparison of PANC-1 cells differentiation with Control SFM, Activin-A and Swertisin. Panc-1 cells cultured in complete media at day 0 which were then subjected to differentiation using SFM/ITS, Activin-A and Swertisin for 10 days. Bright field image of cells under differentiation for day 3rd, day 8th at 5X and 10X magnification respectively and dithizone stained ILCC on day 10th are shown. (C) shows comparative and qualitative insulin immunofluorescence signals in ILCC differentiated from SFM/ITS, Activin-A and Swertisin. Insulin is stained with Alexa-488 labeled antibody showed in red color and nucleus were counterstained with DAPI in blue. Pixel colocalization graphs shows co-localized distribution pattern of insulin and dapi fluorescence in differentiated ILCCs. (D) represents graph for quantification of insulin immunofluorescence per unit cytoplasm from immunostained ILCCs in Control SFM, Activin-A and Swertisin groups. Insulin signal stained with alexa-488 in Swertisin group compared to both SFM and activin-A group. Data was calculated from every single cell in three different frames per slide and expressed as mean ± SEM. *** and ** shows p value less than 0.001 and 0.01 in Swertisin group with respect to SFM and Activin-A group.

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in alexa 488 signals was observed with both Activin-A and Swertisin mediated clusters (Fig 1C), indicated accelerated fate.

Swertisin follows Activin-A mediated pathway for islet differentiation

In order to understand the mechanism of action of Swertisin for islet differentiation, we carried immunoblotting of key transcription factors in endocrine reprogramming from Swertisin induced clusters at day 10^{th}. Cited literature clearly states that Activin-A carried new islet formation via ACT-MEPK-TKK pathway [17–19]. As this mechanism is very well reported, so we tested Swertisin and observed for involvement of MEPK-TKK pathway.

PANC-1 cells in undifferentiated state show high proliferation by persistent ki67 expression and low nestin, PDX-1 and Ngn-3 expression. These cells were found to express high phospho-p38 and depleted basal p-38 (Fig 2A). The presence of high e-cadherin but less n-cadherin expression postulated progenitor nature and undifferentiated state (Fig 2A). Protein lysates of 10^{th} day differentiated islet like clusters, when probed for various transcription factors and MAP kinase signal proteins, revealed substantial inhibitory effect on proliferation rate with low Ki67 expression in both the groups, *i.e.* SFM/ITS, and Swertisin, demonstrating differentiation. However, an elevated expression of nestin in Swertisin clusters but not in other group, confirmed strong endocrine reprogramming with Swertisin. Ngn-3 protein at day 10^{th} was found to be significantly down-regulated in Swertisin induced clusters but remained unchanged in SFM/ITS and undifferentiated cells. Low Ngn-3 protein level indicated that PANC-1 cells with Swertisin are enforced to acquire accelerated endocrine phenotype, so the expression decreases significantly as evident in mature islet cells (Fig 2A).

Further, on 10^{th} day, Swertisin clusters showed high p-38 phosphorylation with basal p-38 compared to SFM/ITS but not with undifferentiated PANC-1. Increased phosphorylation indicates accelerated fate of islet differentiation following p-38 mediated activation, which synchronize with ngn-3 upregulation leading to new functional islet cells (Fig 2A). Comparative statistical protein expression reveal that Swertisin at 10^{th} day able to significantly increase high nestin, pre-requisite for differentiation signals, followed by modest increase in PDX-1 expression, with persistent p-38 phosphorylation and significant Ngn-3 down regulation compared to SFM/ITS, conferring beta cell neogenesis (Fig 2A). This can also be supported by ratio of n-cadherin to e-cadherin, which is critical in order to understand the fate of conversion of progenitor from undifferentiated phenotype to differentiated ones. Swertisin clusters after 10 days showed significantly high n-cadherin to e-cadherin ratio, which depicted, differentiated nature of cells whereas SFM/ITS did not show any change compared to undifferentiated cells (Fig 2B).

Swertisin mode of action involves p-38 phosphorylation and Ngn-3 activation in time dependent manner

Ten-days proteomic analysis highlighted involvement of p-38 but at this time, ngn-3 up regulation was not observed in response to p-38 phosphorylation. Hence, to investigate the expression pattern of ngn-3 in response to p-38 phosphorylation, we did 1–10 days time dependent study for p-38 phosphorylation along with key transcription factors. Immunoblot study from day 1 to day 10 in clusters differentiated with SFM/ITS and Swertisin demonstrated that Swertisin follows differentiation process similar to Activin-A mediated pathway. Samples harvested on each day probed for nestin, ngn-3, pdx-1, phospho and basal-p38 and E-Cadherin (Fig 2C).

We noted that Nestin, a marker for pancreatic progenitor cells in SFM/ITS clusters appears just for initial period, 2–3 days after which it disappears leaving cells undifferentiated, whereas with Swertisin it shoots up by day 2 and remains till day 9, indicating that nestin enforcing these cells to change from precursor type to differentiated cells. The pancreatic duodenal

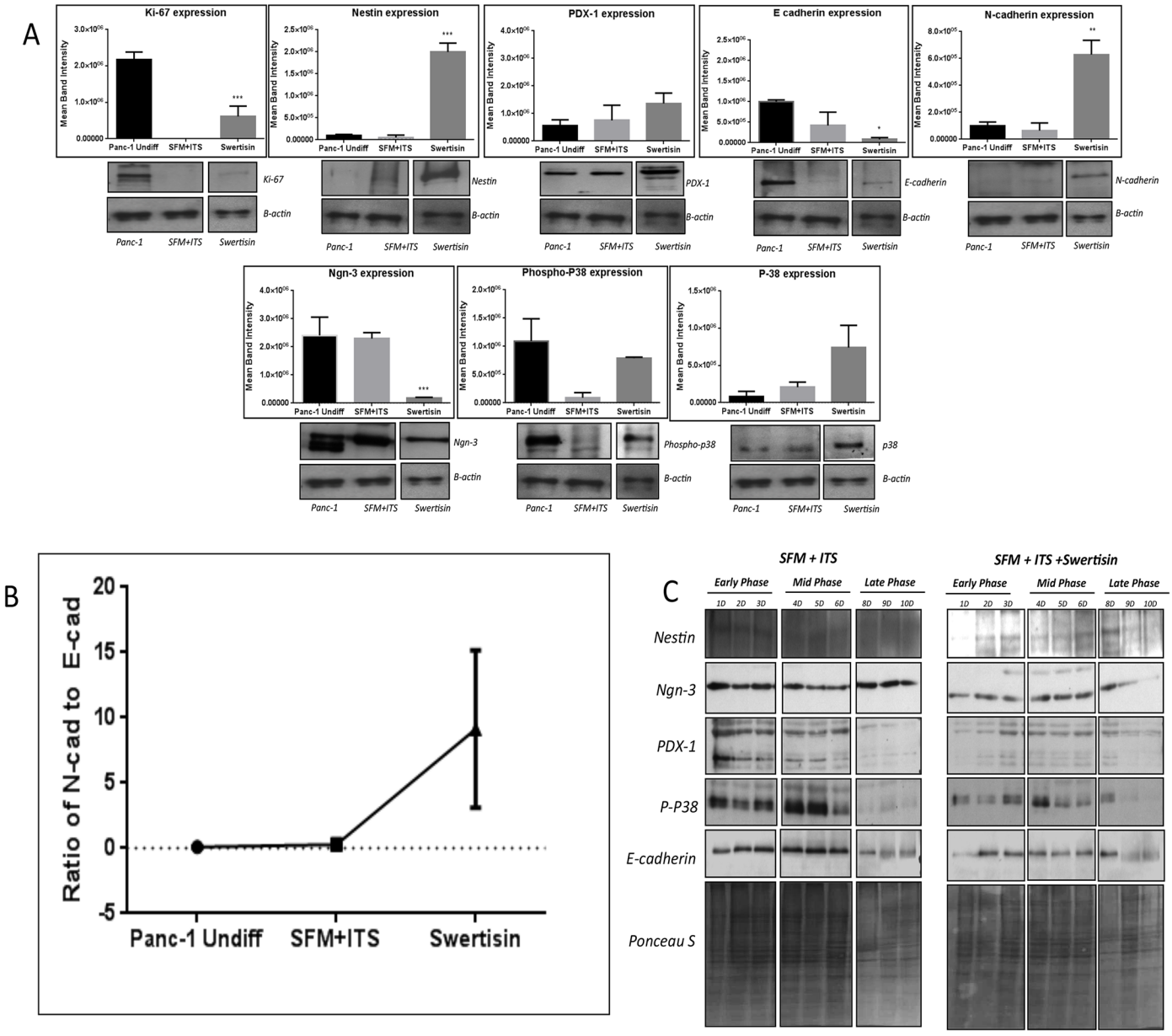

Fig 2. Time course protein profiling and Fate of islet differentiation of Swertisin induced islet differentiation pathway from PANC-1 cells. (A) represents immunoblotting of key parameters that indicate the movement of PANC-1 cells from precursor cells to endocrine islet-like cells, such as stem cell marker Nestin, pancreatic endocrine islet markers Ngn-3 and PDX-1. The activation of p38 MAP kinase to form phospho-P38 and replication marker Ki-67. Protein expression was quantified densitometrically from three independent experiment and expressed as Mean±SEM. *** shows p value <0.001 Vs Panc-1 undifferentiated cells and SFM/ITS. Immunoblotting of E cadherin and N Cadherin demonstrating fate of islet differentiation from undifferentiated PANC-1 cells to endocrine islet-like cells. Graph represents ratio of N-cadherin to E-cadherin depicting transition from precursor sate to differentiated state. Protein expression was quantified densitometrically from three independent experiments and expressed as Mean±SEM. (B) demonstrate characterization of Swertisin induced formation of islet-like clusters from PANC-1 cells in ten days time course. In this experiment shown, cells were harvested each day till ten days and immunoblotted for key parameters that indicate the movement of PANC-1 cells from precursor cells to endocrine islet-like cells in time dependent manner. Cells from control SFM/ITS and Swertisin, harvested from day 1 to 10 were probed for nestin, Ngn-3 PDX-1, phospho-P38 and E-cadherin. (C) shows short-term time course for key proteins implicated in islet differentiation pathway from Swertisin induced clusters. Immunoblotting of nestin, phospo-P38 and Ngn-3 in short-time course manner at 1, 3, 6, and 9 hours induction was performed. Lower images represent graphical representation of movement of protein expression in each group during 1 to 9 hours.

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homeobox gene 1 (PDX-1), a marker of pancreatic endodermal cells remained unchanged up to six days with a modest increase during 7–10 days and same is the case with Swertisin as well. Further, the master regulator gene Ngn-3 expression was strongly upregulated in SFM/ITS from day-2 and persists till 10^{th} day whereas in Swertisin, it elevates right away in early phase day-1 and peaks at mid phase day 4–6 with a declined in latent phase day 9 (Fig 2C), following a typical expression pattern during pancreatic morphogenesis. In parallel, since the effect of Activin-A on Ngn-3 expression is mediated via p38 MAP kinase (Figure A and B in S4 Fig), we also noted that phospho-p38 MAP kinase signal increased by day-2 and remains high till day-6 in SFM/ITS, after which it declines. Similar trend was also observed with Swertisin where phospho-p38 MAP kinase signal increases by day-2, peaks at day-4 leading to activation of Ngn-3 in consequence and then declined by day 8, (Fig 2C). The expression of e-cadherin remain unchanged in SFM/ITS cells till last time point while in Swertisin clusters, e-cadherin started to decline by day 6 and went significantly low till day 10^{th} (Fig 2C).

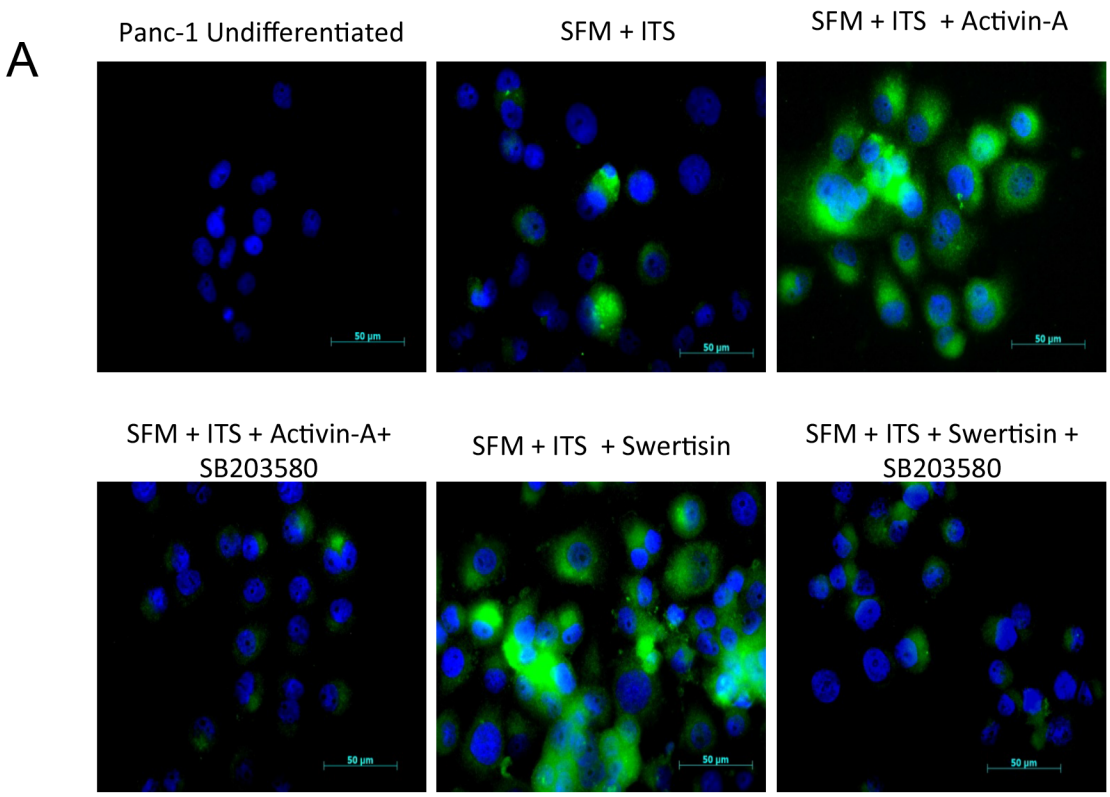
MAP kinase pathway inhibition using specific p38 inhibitor, SB203580 abrogated Swertisin mediated mode of action for islet neogenesis

As proteomic studies done above demonstrated pivotal role of p-38 MAP kinase in islet neogenesis, we confirmed its role using strong inhibitor of MAP kinase pathway. We observed that Panc-1 cells when differentiated with either Activin-A or Swertisin, showed deep cytoplasmic insulin immunofluorescence staining (green). Undifferentiated Panc-1 cells failed to stain for insulin, while those of SFM/ITS showed little insulin positive stained cells. In order to confirm, that Swertisin does mediate islet differentiation *via* MAP kinase pathway, similar to Activin-A, we allowed cells to differentiate in presence and absence of specific p-38 MAP kinase inhibitor SB203580 along with Activin-A and Swertisin. We observed significant reduction in insulin positive cells when Activin-A clusters were differentiated in presence of SB203580 (Fig 3A). Similarly, Swertisin induced clusters also fail to synthesis insulin in presence of SB203580 (Fig 3A). Quantitative measurement of insulin fluorescence per unit cytoplasm clearly demonstrated significant retardation in insulin expression in both Activin-A and Swertisin mediated clusters with SB203580 confirming the involvement of MEPK-TKK pathway in islet differentiation, which gets abrogated upon inhibition (Fig 3B).

Isolated primary cultured mouse intra-islet pancreatic progenitor cells showed islet differentiation with Swertisin induction

Demonstrating the mechanism of action for new islet formation from small molecules like Swertisin using cell line model system PANC-1, enlighten involvement of MEPK-TKK proteins. It is more relevant to reconfirm these facts in primary cultured cell model for pancreatic progenitor cell system. Hence, we isolated mouse intra-islet pancreatic progenitor cells and developed a primary culture. We successfully established a stable cell system which showed initial populate of fibroblast to epithelial morphology by passage-3, but these cells later stabilized and formed pure colonies by passage-5 and complete homogeneous cell population by passage-7-11 (Figure A in S1 Fig). We further characterized these cells for their progenitor nature and found them positive for nestin, vimentin, ngn-3 and pdx-1 expression. Importantly we could show that these cells were negative for insulin staining at this time, which confirms their undifferentiated state (Figure A in S1 Fig).

We further confirmed the presence of progenitor marker and monitored protein expression using immunoblotting in these cells at different passages right from isolation till it develops into stable cell line. Nestin a marker of pancreatic progenitor, was very weekly expressed at passage-5, increased gradually by passage-7, 9 and highly peaked at passage 11 and remained



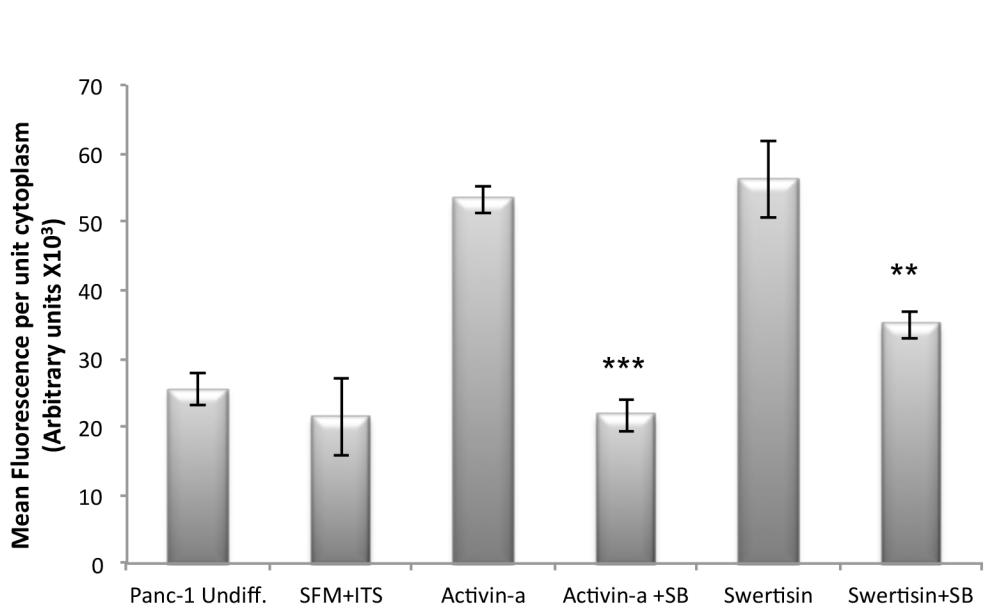


Fig 3. Insulin fluorescence and Insulin content per unit cytoplasm quantification in Swertisin induced islet-like clusters derived from PANC-1 cells with activin-A and MAPKinase Inhibitor. (A) shows fluorescent images for insulin expression in islet differentiation pathway inhibited using p38 MAP kinase inhibitor SB-203580 added in conjunction with Swertisin throughout ten days. The islet-like clusters were immunostained for insulin (green) on 10^{th} day. Nuclear DNA was stained with DAPI (blue). (B) represents insulin content in islet like clusters after p-30 MAPK pathway inhibition. Graph represents insulin fluorescence quantification per unit cytoplasm in differentiated cells with and without inhibition of MAPK pathway using p38 MAP kinase inhibitor SB-203580 added in conjunction with Swertisin throughout ten days. Data is represented as Mean±SEM. *** and ** shows p value <0.001 and 0.01 Vs Activin-A and Swertisin alone groups respectively.

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constant thereafter. E-cadherin, expressed by stem/progenitor nature cells, was also found to be increase in gradual manner from passage 7 till 11. Similarly, Vimentin showed increased expression pattern by passage7-11. More likely, Basal expression of pdx-1 and ngn-3 was found right from passage 5 till passage 9, which signifies that cells are maintaining their progenitor state. Extremely low expression of n-cadherin depicted undifferentiated state of these cells in all passages (Figure B in S1 Fig).

Swertisin acts via p-38 MAP kinase and involves Smad2/3 transcriptional regulation

Mouse intra-islet progenitor cells were subjected to islet differentiation with SFM/ITS, Activin-A and Swertisin. Islets like clusters were formed with both Activin-A and Swertisin in 8-day protocol. Clusters thus formed were found positive for deep crimson red DTZ staining showed presence of insulin. The islets generated from each group were immunostained for insulin and glucagon. SFM/ITS did not show evident insulin (green) and glucagon (red) staining, while both Activin-A and Swertisin clusters showed intense insulin and glucagon staining (Fig 4A).

Further, we observed immunoblotting profile of key proteins involved in islet differentiation pathway on 10^{th} day and reconfirmed the role of p38 MAPK using its inhibitor SB203580. Undifferentiated mIPC and SFM/ITS clusters showed high e-cadherin but no n-cadherin. Also ngn-3 was not activated in spite of high p38 phosphorylation (Fig 4B). On other side Activin-A and Swertisin differentiated clusters showed presence of n-cadherin followed by elevated ngn-3 expression triggered by p38 phosphorylation which goes depleted by this time. More interestingly, when clusters differentiated with SB203580 were probed, we found that n-cadherin was lost by this time and ngn-3 failed to upregulate with low p-38 phosphorylation (Fig 4B) these observations again highlighted that Swertisin mediate islet differentiation involving p38 MAPK through MEPK-TKK pathway

Further we attempted to monitor downstream signaling of p-38, involving Smad protein complex 2/3 and Smad 7 playing pivotal role in differentiation signaling and control of transcription factor expression like ngn-3. We compared SFM/ITS, Activin-A and Swertisin together for Smad2/3, Smad-7 in response to phospho-p38 in time dependent manner from day 0 till day 8. SFM/ITS clusters showed high Smad 2/3 and Smad 7 expression which remain elevated till day 8, whereas Activin-A and Swertisin both molecules showed initial Smad2/3 levels, which goes down progressively over differentiation process and maturation along with steep decline in, Smad-7 right from day-2 with p-38 phosphorylation (Fig 4C). In continuation, we also observed levels of Erk1/2 along with p-38, where SFM/ITS group showed high basal Erk1/2, which remains continued till day 8. However both activin and Swertisin showed gradual depletion in erk1/2 levels right from the beginning, indicating progression of differentiation by possibly phosphorylation of erk1/2 in response to phospho-p38. Further, we also observed pax-4 expression for maturation of progenitors into beta cells lineage. SFM/ITS showed a very delayed pax-4 expression at day 6–8. However activin-A could show pax-4 increase by day4, while Swertisin triggers pax-4 increase right from day2 till day 8 (Fig 4C). These evidences confirm that p-38 proceed with Smad protein complex recruitment as reported with Activin-A mediated TGF-beta signal transduction during pancreatic morphogenesis [8].

MAP Kinase along with Smad proteins signals facilitate endogenous islet differentiation by Swertisin in-vivo

Ppx has been reported to involve beta cells replenishment by islet neogenesis from stem/precursor cells. In present experiment we made an attempt to investigate the mechanistic action of

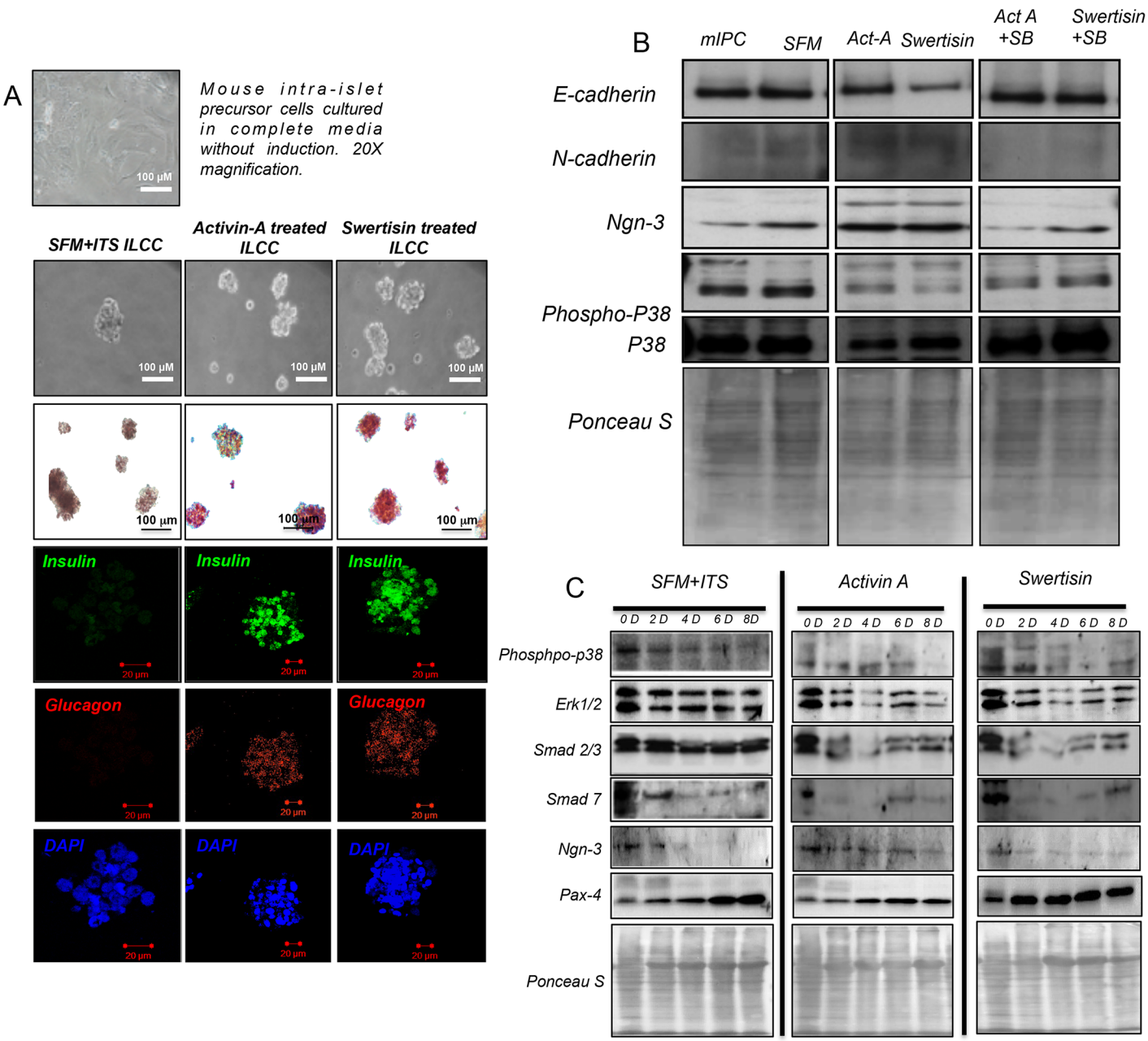

Fig 4. Differentiation of mouse intra-islet progenitor cells and immunoblotting of key transcription factors and islet markers under differentiation with activin, swertisin and presence of p-38 MAPK inhibitor. (A) depicts mIP cells cultured in complete media at day 0 which were then subjected to differentiation using activin-A and swertisin for 10 days. Bright field image shows, cells under differentiation on 8th day at 20X magnification, and dithizone stained clusters on day 10th. A fluorescent image represents immunostaining for insulin (green) and glucagon (red) in clusters from SFM/ITS, activin-a and swertisin groups. DAPI was used as nuclear stain. (B) shows immunoblotting of E-cadherin, N-cadherin, Ngn-3, P-p38, Native p-38 MAP kinase pathway proteins in presence and absence of MAP kinase pathway inhibitor SB203580. Ponceau S stain blot was shown as loading control. (C) shows immunoblotting of key differentiation pathway parameters that indicate the conversion of Mouse intra-islet progenitor cells into islet like clusters. Key transcription factors and MAP Kinase pathway proteins like P-p38, Erk1/2 Ngn-3, Pax-4, and Smad proteins under differentiation were monitored. Beta actin was used as loading control.

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Swertisin induction for new islet formation mediated through MEPK-TKK pathway, under *in-vivo* condition in surgically induced pancreatectomised mice.

We observed that mice undergone 70% Ppx with and without Swertisin did not demonstrate any change in body weight during three days post PPx, compared to sham operated control mice (Data not shown). An increasing trend in remnant pancreas tissue weight has been observed in Swertisin treated animals on 3rd day, notifying regeneration of small fraction of new pancreas, although it is not statistically significant compared to control animals (Figure B in S2 Fig). Fasting blood glucose also remained unchanged in both the groups over three days (Figure C in S2 Fig).

In order to confirm the islet differentiation, mRNA expression for various islet specific transcription factors was observed using RT-PCR. Semi quantitative gene expression data revealed that Ppx alone animals favored activation of pancreatic progenitor gene–PDX-1 and doesn't reach to endocrine fate commitment until 3rd day post Ppx. On the other hand, Swertisin treated Ppx mice identified low PDX-1 but more Ngn-3 expression suggesting early induction and endocrine fate regeneration signals (Figure A and B in S3 Fig). None of the animal group had shown nestin expression, but Swertisin treated PPx mice reveled significantly elevated insulin gene mRNA transcripts compared to Ppx alone (Figure B in S3 Fig).

More importantly, protein lysates extracted from regenerated pancreatic tissues on 3rd day were probed for islet, MAP Kinase and Smad transcriptional pathway proteins. In western blot analysis we found that Ppx alone animals fails to induce nestin expression as noted in mRNA data earlier, whereas Swertisin treated Ppx animals persist some nestin expression leading to early differentiation signals (Fig 5A). Further the marker of ductal progenitor cells Cytokeratin-19 (CK-19) was highly upregulated in Ppx alone mice depicting ductal fibrosis (duct cell proliferation) which was significantly down regulated in Swertisin treated animals. E-cadherin which is known to express more in progenitor cells compared to differentiated cells has been observed in high proportion in Ppx alone animals but low expression was recorded in Swertisin treated mice. Interestingly, at protein levels we did not find any significant change in PDX-1 expression in both the groups, but key master regulator ngn-3 expression was significantly upregulated in Swertisin treated mice compared to untreated ones. This dramatic increase in ngn-3 expression in Swertisin treated mice confirmed the stated role of p38 MAPK as a trigger for MAPK-TKK pathway and neuro-endocrine differentiation signals. To further confirm this, the downstream TF like p-Smad2, 3 and 7 were analyzed. When compared, we found Ppx alone mice expressed significantly high Smad 7, whereas samd-2 phosphorylation, which is a prerequisite for induction of islet differentiation pathway, was high in Swertisin treated animals (Fig 5A).

Activation of MAP Kinase leading to Smad regulatory mechanism is responsible for new islet cells mass in regenerated pancreas

We then observed and confirmed p-38 mediated activation of ngn-3 expression leading to islet neogenesis in regenerated excised pancreatic tissues from Ppx alone and Swertisin treated animals (immunohistochemistry) by probing various markers for islet differentiation pathway and Activin-A signaling pathway. In Ppx tissues we found that nestin expression (green) was randomized in some patches near acinar tissue, but few islet cells showed nestin positivity as well. None of these areas showed ki67 expression (red) indicating less proliferation of acinar and islet cells. A persistent proliferating population of ngn-3 positive (green) cells was observed with dual ki67 staining (red). Moreover Ppx animals also showed intense Ck19 (green) staining in epitheloid ductular structures. Most important observation was that PPx animal tissue had deep smad-7 (green) staining and very weak p-Smad 2 (red) staining. The presence of low c-peptide with few scattered glucagon positive cells and compact size islet were observed indicating slower differentiation process in newly regenerated untreated (Sham) mouse pancreas (Fig

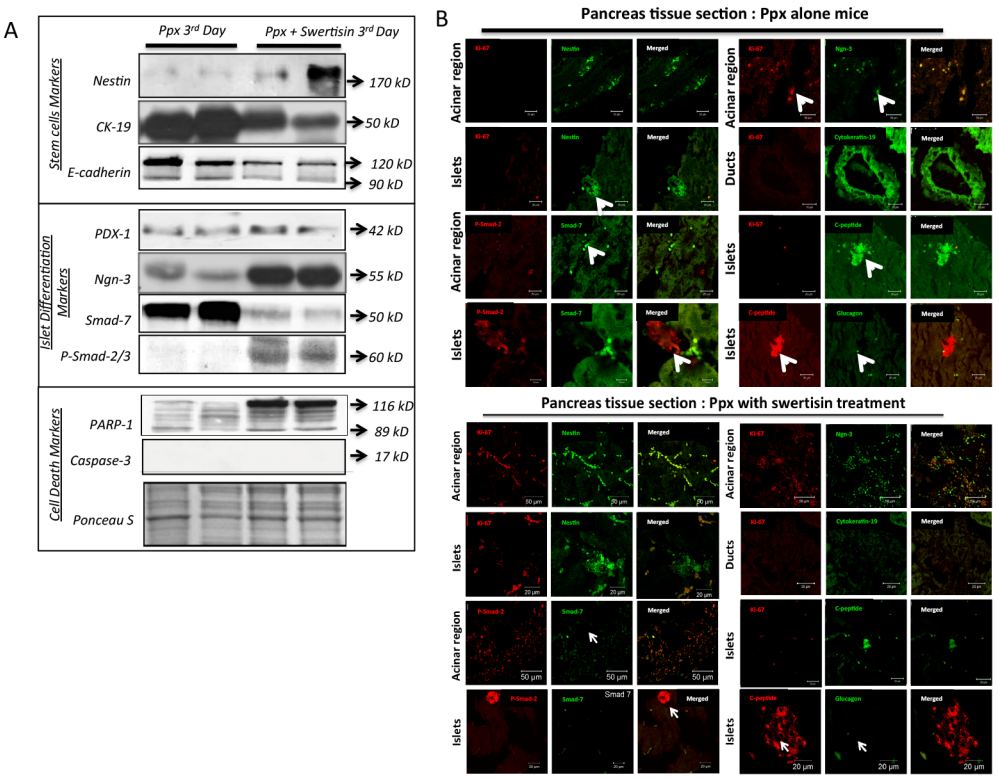

Fig 5. In-vivo analysis of molecular mechanism by Swertisin differentiation in Ppx mice model. (A) confocal images from regenerating pancreas for assessment of various islet transcription factors and signaling proteins of TGF-beta pathway from tissue of Ppx sham and Swertisin treated animals. Various markers like Ki67, Nestin, Ngn-3, CK19, p-smad-2, Smad-7, Insulin and Ck-19 were probed and analyzed. (B) showed western blot profile of stem cell markers, key transcription factors in islet differentiation pathway and cell death markers in PPx Sham and Swertisin treated animal pancreatic tissues.

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5B). On the other hand, Swertisin treated animal tissue showed large number of dual stained population of ki-67 and nestin within both acinar and islet tissues. These sections also showed low Ck19 staining in ductular tissues, but enormously high p-smad-2 staining within acinar and islet area. The staining of smad-7 was lower in treated animals suggesting presence of few progenitor nature cells with faster differentiation (Fig 5B).

Discussion

In the present study, we have reported that Swertisin treatment promoted the differentiation of human and mouse pancreatic progenitor cells mediated through MAP kinase pathway (MEPK-TKK pathway) similar as of Activin-A and could be inhibited for terminal differentiation into endocrine cells in the regenerating pancreas if treated with MAP kinase specific inhibitor SB203580.

Pancreas (organ transplant) or Cadaveric islet transplantation treatment strategies have many limitations [20]. Stem cell to islet differentiation provides alternative approach to cure both Type-1 and Type-2 disease [21]. Currently efforts are being made to generate insulin-producing cells from stem cells or tissue specific progenitor cells [22, 23]. Very few reports are there which show pancreatic regeneration using herbal extract or compounds. One of such study done by Kojima et al. showed insulin positive cell differentiation from AR42J cells using conophylline isolated from Ervatamia microphylla [24].

Enicostemma littorale is one such anti-diabetic plant, which we have examined for its capacity of promoting islet neogenesis reported earlier [14, 15]. *In-vitro* formation of islet-like cell clusters containing both β and α cells were observed, when treated with herbal bioactive agent Swertisin isolated from *E. littorale* [15]. This led us to investigate the molecular mechanism of this potent bioactive compound "Swertisin" for its effective islet cell differentiation property. We hypothesized that Swertisin might differentiate progenitor cells undergoing pathway same as that of Activin-A via MEPK-TKK pathway. To test the mode of action of Swertisin, we used human pancreatic progenitor panc-1 cells and mouse intra-islet primary cultured pancreatic progenitor cells. We compared the crucial transcription factor expression playing inevitable role in islet neogenesis with the clusters generated from both Swertisin with Activin-A in time dependent manner. Exogenous Activin-A treatment increases the proportion of insulin cells in the developing chick pancreas [25]. Activin A also induces differentiation of human fetal pancreatic endocrine cells [26]. Previously few groups have shown that panc-1 cells and primary cultured islet progenitor cells could differentiate into islet like clusters using Activin-A [27, 28], so it become evident to investigate the mechanism of action in new islet generation targeting Activin-A mediated TGF-beta pathway.

In our experiment, Swertisin effectively differentiates panc-1 cells and mIP cells like Activin-A. Further we demonstrated that within ten days of treatment with Activin-A and Swertisin, islet-like clusters were formed that could stain with DTZ and immunostain for insulin and glucagon both. Comparative quantification of insulin content per unit cytoplasm during differentiation in clusters postulated the fact that Swertisin demonstrated, even more efficient in differentiating capacity compared to Activin-A. Cells under differentiation stopped proliferation and showed significant decline in Ki-67 expression According to the literature, islet differentiation pathway starts with either nestin progenitor cells [29, 30] or by activation of master key gene ngn-3 [31, 32]. Nestin was expressed at different levels in the acinar component, as well as in ductal structures and islets to some degree. We have also noted that Nestin, a key marker in islet differentiation signaling, peaks at early time and subsequently declined at later time in differentiation, with strong up regulation of Ngn-3 between mid times in Swertisin mediated clusters from panc-1 and mIP cells. Number of investigators used pdx-1 as candidate master regulator in islet generation where pdx1 protein directly binds and activates the insulin promoter [33], and at least one acinar enzyme gene [34]. Further, Baeyens et al., in 2006 demonstrated that *in-vitro* growth factor stimulation could induce recapitulation of an embryonic endocrine differentiation pathway in adult dedifferentiated exocrine cells via ngn-3 ectopic expression and over activation [32]. In our study we found a very steady expression of pdx-1 in differentiated clusters from start to end, demonstrating no significant impact of pdx-1 over differentiation process. Moreover ngn-3 was the key factor, which showed high upregulation both in cell line and primary culture system. Swertisin, showed high ngn-3 cells in response to p-38 phosphorylation, which highlighted the pivotal role of p-38 MAP Kinases in this process.

Earlier reports demonstrated that Activin-A a member of TGF-β family of proteins that helps in growth and differentiation of β-cells [8, 26], promotes expression of pro-endocrine gene ngn-3 by upregulating p38 MAP kinase [24] through Smad 2/3 phosphorylation and Smad 7 down regulation [8]. In the present study the signaling pathway of both Activin-A and Swertisin is found to be mediated through pro-endocrine gene ngn 3 triggered by of p38 MAP kinase phosphorylation [24], we then confirmed whether SB203580, an inhibitor of p38 MAPK could abolish islet-promoting action. With inhibition of MAP kinase signaling, Activin-A failed to form islets, and the same was observed with Swertisin in both human and mouse cell systems. These facts confirmed that Swertisin do form islets mediated by ACT--MEPK-TKK pathway. Further when looked downstream to p-38, Smad protein showed high

Smad2/3 phosphorylation and reduced Smad 7 expression *in-vitro* and *in-vivo* experiment both with Activin—A and Swertisin.

Ppx is well known model for the study of β-cell regeneration. The mechanism by which regeneration occurs in this model has been controversial, with some claiming that islet and β-cell neogenesis is important, while others claim that β-cell replication is predominant [35, 36]. The process and mode of regeneration efficiency that follows with Ppx injury depends on the degree of surgical insult. In 30–50% Ppx, β-cell mass expansion is mainly due to replication of pre-existing β-cells, but no ductal cell proliferation or Ngn-3 induction. However with 70–90% Ppx, the β-cell mass expansion has been attributed to neogenesis with the proliferation of putative ductal precursors in main pancreatic ducts, and induction of Ngn3 expression. Also there is a wave of proliferation initiated in main duct, which is followed in smaller ducts and finally in islet β-cells, with transient up-regulation of PDX-1 in ductal cells along with nestin and ngn-3 [37]. Also it is interesting to note from literature that activin is upregulated in duct cells following partial pancreatectomy and streptozotocin injection, suggesting that activins might be involved in the initiation of β-cell neogenesis following distinct stimuli in adulthood [38, 39]. On this basis we again examined and reconfirmed the mechanistic action of Swertisin in *in-vivo* condition with 70% Ppx mice model. Seventy percent Ppx animals were treated with single injection of Swertisin in pancreas on day 0, showed high ngn-3 expression by 3rd day with elevated p-samd2 and low samd-7, indicating ACT-MAPK pathway activation. Moreover less e-cadherin and ck-19 expression again reconfirms low ductal cell proliferation, opposite of which was observed in sham operated control animals. Altogether, our *in-vivo* data does present evidences in accordance with earlier *in-vitro* observation made by us [14, 15].

We therefore collectively postulate an underlying mechanism of action for Swertisin mediated by nestin overexpression triggering MAP kinase pathway that involve p-38 MAP kinases and Smad proteins. These phosphorylated proteins recruiting a complex of Smad protein (2/3 and 4) induce differentiation while Smad-7 maintaining progenitor state in these cells. Smad complex then go and bind to DNA for regulating and activation of important key regulatory transcription factors such as ngn-3 (could be one target) and facilitate endocrine reprograming under MAP kinase signals (Fig 6). Elevated neurogenin-3 expression work as a driving force for these cells to undergo endocrine reprograming and form new islet like cell clusters via p-38 MAPK signals which gets abrogated by p-38MAPK inhibitor, when added (Fig 6).

Conclusion

In the present study, we first time reported the mechanistic action of a potential natural islet neogenic agent Swertisin and demonstrated this islet forming action by tracing expression of crucial transcription factors and MAP kinase pathway with and without inhibition using human pancreatic progenitor panc-1 cells, mouse intra-islet progenitor and 70% Ppx mouse models. Swertisin differentiate stem/progenitor cells into insulin producing cells via MEPK-TKK pathways followed by TGF-β (Activin-A mediated) pathway for new islet clusters formation both *in-vitro* and *in-vivo* via nestin driven p-38 phosphorylation in-turn regulating ngn-3 overexpression. Moreover, the information of endogenous islet neogenic potential seems to be very useful in designing newer therapeutic approaches and target molecules, governing TGF-β mediated signaling pathway to treat diabetes mellitus.

Material and Methods

Cell culture maintenance

Human Panc-1 cells, as described earlier by Lieber M et al. [40], were obtained from Dr. Girish M Shah's Lab. Universite Laval, Quebec, Canada (as generous gift) and maintained in high

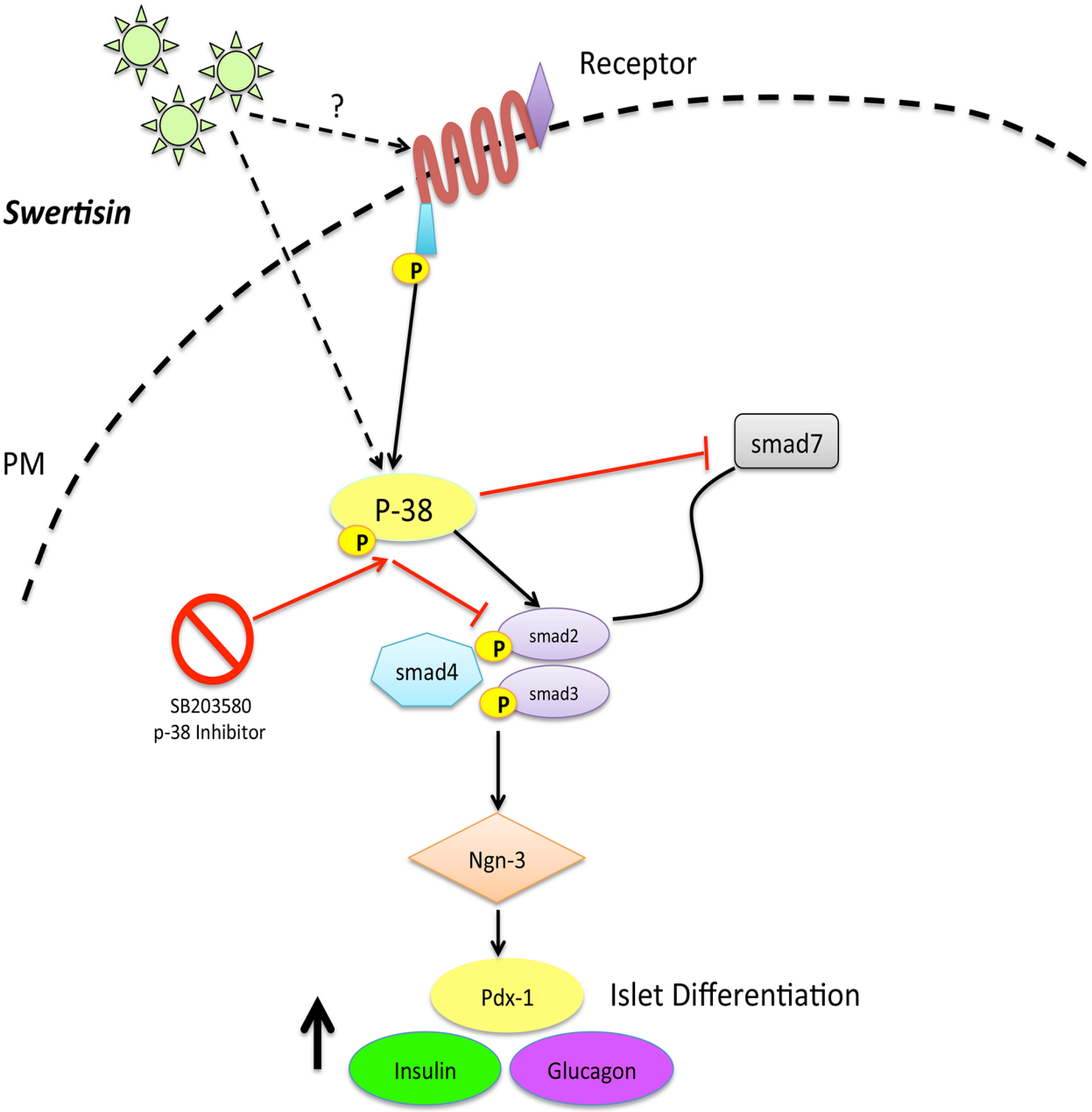

Fig 6. Proposed Mechanism of action of Swertisin mediated Islet Differentiation. Panel shows proposed mechanistic action pathway for islet neogenesis being followed by Swertisin for new islet cell differentiation.

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glucose DMEM supplemented with 10% Fetal Bovine Serum. Cultures were maintained in 95% air/5% CO2 at 37°C, and the medium was replenished every alternate day. Cells were regularly monitored for subculturing and trypsinized with 0.5% TPVG (Sigma Aldrich, USA) at 80% confluency.

Isolation and establishment of mouse intra-islet progenitor cells

Mouse intra-islet progenitor cells were isolated from fresh islet preparation followed by digestion of whole mouse pancreas. Mouse pancreata were dissected and subjected to collagenase

digestion (Sigma Aldrich, USA), as described previously [41]. Two pancreata were used per isolation, and islet isolations were performed for each time point in the study. Purification was achieved and the final preparation was confirmed with 90% dithizone-positive structures. Freshly isolated islets (5000 islets per 25 cm2 flask) (Corning, Fisher Scientific, Canada) were placed and cultured in RPMI-1640 complete media with 10% serum (Gibco, ON, Canada) for 24 hours and then shifted to DMEM complete media with 10% serum (Gibco, ON, Canada) to promote monolayer formation. Cultures were maintained in 95% air/5% CO2 at 37°C, and the medium was renewed every alternate days. The monolayers were subcultured when the cells had grown to near confluence. Representative islet cultures were examined immediately after isolation (day 0), and the derived monolayers were examined at 1, 5, 7, 9 and 11 passages of the culture period in the subsequent investigations.

Differentiation of Panc-1 and mIP cells into islet like clusters

The cells were allowed for differentiation in presence of Serum free medium, Activin-A and Swertisin as previously described [14]. Normal Panc-1 cells and mIP cells were differentiated using Activin-A at 20 ng/ml and Swertisin at 15μg/ml concentrations as differentiating factors in a eight day differentiation protocol with insulin (5μg/ml) transferrin (5μg/ml) and selenite (5ng/ml) cocktail (Sigma, Aldrich USA). In order to better comprehend the differentiation procedure of islet neogenesis with Panc-1 and miP cells, differentiation in presence and absence of SB203580 (Inhibitor of p-P38-MAPK) was performed to understand role of p38 MAP Kinase. In one of the groups Activin-A and Swertisin was added in combination with SB203580, a specific inhibitor of p-38 MAP Kinase at 10μM concentration.

Immunohistochemistry/Immunocytochemistry

In order to understand the mechanism of Swertisin action in *in-vitro* generated clusters form Activin-A and Swertisin and in regenerated pancreas after 70% pancreatectomy, various islet and key differentiation protein markers were probed immunochemically. Undifferentiated Panc-1 cells and mIP cells were also assessed for their progenitor nature in similar way. Immuno-cyto/histo-chemistry of the following protein markers: Ki-67, Nestin, P-Smad-2, Smad-7, C-peptide, Glucagon, Ck-19, Pdx-1 and Ngn-3 were done. Insulin and Glucagon immunocytochemistry staining was also done to characterize clusters obtained after differentiation from panc-1 cells and mIP cells. Briefly, growing cells or clusters were fixed with 4% PFA for 10 min at 4°C and washed once with PBS, thereafter permeabilized with 0.1% triton X-100 solution in PBS form 5 min at 4°C. Once permeabilization done, samples slides were blocked with blocking solution containing 0.5% BSA with 4% FBS in PBS for 1 hour at room temperature. Slides were then probed with respective primary antibody at desired dilution as shown in S1 Table. Followed with 3 time washes in wash buffer (one tenth of blocking buffer), slides were labeled with fluorescent dye conjugated secondary antibody (dilution shown in S1 Table). Nuclear counterstaining was done with DAPI at 300nM and finally mounted with vectashield mountant for imaging on fluorescent microscope. Images were captured with Nickon TE200S inverted fluorescent microscope and data was analyzed with NIS element Advance version software (Nickon, Japan).

Protein extraction and Western blotting

Protein samples from various cells differentiated clusters and dissected pancreatic tissue on day-3 post Ppx was harvested and homogenized. Followed with protein isolation, total protein was estimated, which was further analyzed by western blot profiling of the following proteins: Nestin, E-Cadherin, N-cadherin, PDX-1, Neurogenin-3, Phospho-P38, P-38, Smad-7,

P-Smad-2/3, Parp-1 and Caspase-3. Cells/ clusters/ tissues were lysed with urea containing lysis buffer (1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.). Protein estimation in all samples was carried out using Bradford reagent according to manufacturer's suggestions (Biorad, USA). Cell lysates (50μg) were separated on Polyacrylamide gel using Mini-tetrapod electrophoresis system (Biorad, USA) and transferred onto nitrocellulose blotting membrane (Thermo Inc.). Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in PBS). Dilutions of primary antibodies used, against various proteins, listed in S1 Table. Primary antibodies were added to blots and incubated overnight at 4°C. Anti-rabbit and Anti-mouse IgG conjugated with HRP were used to develop the blots using Ultra sensitive enhanced chemiluminiscence reagent (Millipore, USA) and images were captured on Chemigenious gel documentation system (Uvitech, Cambridge).

RNA extraction, First Stand c-DNA synthesis and semi quantitative Reverse Transcriptase PCR (RT-PCR)

Pancreatic tissue of control and treated mice with Swertisin on the 3rd day post Ppx were dissected and subjected to RNA isolation, first stand c-DNA preparation followed by gene expression profiling by semi quantitative RT-PCR for Nestin, PDX-1, Ngn-3, INS and G6PDH. Total RNA was isolated with TriSoln (Sigma Aldrich, USA) and quantified on Shimadzu Nanospectrophotometer (Shimadzu, Japan) at lambda 260 and 280 nm. RNA integrity and purity was checked on a 1.5% denaturating agarose gel electrophoratically. Followed this 1 mirogram of RNA was reverse transcribed into cDNA using first strand c-DNA synthesis kit (Fermentas INC., USA) as per manufacturing instruction manual. RT-PCR was done with optimal conditions for various genes (see S2 Table) by running gradient PCR performed with a range of annealing temperature from 51–60°C. One μl of cDNA products was used to amplify genes using Fermentas 2X master mix containing 1.5 μl Taq Polymerase, 2mM dNTP, 10X Tris, glycerol reaction Buffer, 25mM MgCl2, and 20pM appropriate forward and reverse primers for each gene (for sequence and details see S2 Table). PCR products were then resolved on a 1.5% Agarose gel (Sigma Aldrich, USA) and visualized with ethidium bromide staining. Images were analyzed using Alpha Imager software (UVP image analysis software systems, USA).

Animals

Male balb/c mice, 3–4 weeks old, weighing around 25–30 grams, were used for islet isolation for the generation of mIP cells and 70% partial pancreatectomy to understand the mechanism of pancreatic regeneration process *in-vivo* with Swertisin treatment. This study was carried out in strict accordance as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, India (CPCSEA). Post experiment animals were euthanatized using xylazine (10 mg/kg) and ketamine (150mg/kg) injection followed by cervical dislocation ensuring death. The protocol was approved by the Committee on the Ethics of Animal Experiments from our institution- The Maharaja Sayajirao University of Baroda, Gujarat, India.

Partial Pancreatectomy

Seventy percent partial pancreatectomy was performed according to Bouwens et al., and Bonner-weir et al. [22, 29]. Briefly, Mice were anesthetized by administration of ketamine and Xylazine (100 mg/kg and 50 mg/kg bwt; i.p.). Hairs were cleaned and the abdomen was opened through a left lateral incision. The entire splenic portion and most of mesenteric portion of the pancreas was surgically removed, resulting in ~70% pancreatectomy, confirmed by weighing

the removed and remnant portions. Sham operation was performed, by opening the abdomen while leaving the pancreas intact. The incision was closed using 4–0 silk treads for the peritoneum and sutured back for the outer skin as well. Animals were given injections of pain reliever for 3 days and wounds were dressed with tropical skin ointment.

Supporting Information

S1 Fig. Isolation and Immuno-characterization of Mouse Intra-islet pancreatic progenitor cells. (Figure A) demonstrate establishment of mouse intra-islet progenitor cells isolated using collagenase type-5 digestion. Purified islets were cultured in condition growth medium and passaged in subsequent generations. Cells at passage no 3, 5 and 7 were captured in bright field image. Immunofluorescence staining shows ngn-3 (TRITC-red), vimentin (cy5-red), nestin (TRITC-red), pdx-1 (FITC-green) and insulin (FITC-green). DAPI was used to counterstain nuclei (blue). (Figure B) shows immunoblotting of key parameters that indicate the establishment of Mouse intra-islet progenitor cells from isolated islet cells in passaging. Key stem/progenitor markers like Nestin, E-cadherin, mesenchymal stem cell marker Vimentin pancreatic endocrine islet markers Ngn-3 and PDX-1, and cell differentiation marker N-cadherin was used. Ponceau S stain blot was shown as loading control.
(TIF)

S2 Fig. Swertisin molecule structure, Pancreatic Tissue weight and Fasting glucose in Ppx animals. (Figure A) showing Swertisin molecular structure. (Figure B) graphs demonstrate change in pancreas weight calculated on 3rd day post sacrifice and (Figure C) shows fasting blood glucose in sham operated and Swertisin treated animals.
(TIF)

S3 Fig. Gene Expression and histology of Ppx mice tissues. (Figure A-B) shows gene expression data for various islet specific markers in these regenerating pancreatic tissues. Quantitate mRNA expression of genes expressed in post Ppx with and without Swertisin treatment was analyzed. All data seta are represented as mean ± SEM and calculated from 3 independent animal observations. *** and ** represents p value < 0.001 and 0.01 Vs Ppx animals. (Figure C) shows images of pancreas regeneration at 10 day, demonstrating beta cell regeneration
(TIF)

S4 Fig. Immunoblot profile of Activin-A mediated islet differentiation pathway. (Figure A) shows western blot profile of activin-A mediated islet differentiation in panc-1 ILCC in time dependent manner. (Figure B) shows protein profile of key differentiation markers in short time 0–9 hours.
(TIF)

S1 Table. List of antibodies used in IHC/ICC and immunoblot. Shows list of primary antibodies used in ICC, IHC and western Blot experiments with particular details for each experiment like specificity, dilution factor, molecular weight etc.
(DOCX)

S2 Table. List of primer sequences used in RT-PCR. Shows list of forward and reverse primer sequences on all genes used in RT-PCR experiments along with melting point and amplicon size for each gene.
(DOCX)

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Author Contributions

Conceived and designed the experiments: ND GMS Sarita Gupta. Performed the experiments: ND AS NP Shivika Gupta AD. Analyzed the data: ND GMS RRB Sarita Gupta. Contributed reagents/materials/analysis tools: ND GSM Sarita Gupta. Wrote the paper: ND Sarita Gupta.

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RESEARCH ARTICLE

Anti-apoptotic and cytoprotective effect of *Enicostemma littorale* against oxidative stress in Islets of Langerhans

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ABSTRACT

Context Oxidative stress induces apoptosis within Islets of Langerhans in diabetes mellitus (DM). *Enicostemma littorale* blume, herb of the Gentianaceae family is used as an anti-diabetic agent across rural India.

Objective This report demonstrates potent anti-apoptotic and cyto-protective activity of *Enicostemma littorale* MeOH extract (EL MeOH ext.) against 50 μM H_2O_2 in isolated rat Islets.

Materials and methods In this study, the whole plant methanolic extract of EL with doses 0.25–4 mg/mL each for the preincubation duration of 0.5–4 h against 50 μM H_2O_2 were tested for optimum protective dose and time by Trypan blue dye exclusion assay. Islet intracellular reactive oxygen species (ROS) was quantified by DCFDA staining and cell death using PS/PI & FDA/PI staining. Further, comet assay, biochemical assessment of caspase-3 and antioxidant enzyme activities along with immunoblotting of PARP-1, caspase-3, TNF-α activation and p-P38 MapK (stress kinase) induction was performed.

Results The optimized dose of EL MeOH ext. 2 mg/mL for 2 h was used throughout the study, which significantly decreased total Intracellular ROS and cell death. Further, caspase-3 activity, PARP-1 cleavage, p-P38 MapK (stress kinase) activation and TNF-α levels, which had been significantly elevated, were normalized. Antioxidant enzymes like catalase, superoxide dismutase, reduced glutathione and glutathione peroxidase, along with Comet assay, demonstrated that pretreatment with EL MeOH ext. can augment antioxidant enzyme activities and protect from DNA damage.

Discussion and conclusions Significant anti-apoptotic and cyto-protective effects were mediated by EL with Islets of Langerhans subjected to oxidative stress-induced cell death.

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Introduction

Reactive oxygen species (ROS) are constantly generated under normal metabolic conditions, as a consequence of aerobic metabolism. They are particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates and lipids in a destructive manner which leads to various pathophysiological diseases, including diabetes, Parkinson's, Alzheimer, and retinal degeneration (Bonnefont-Rousselot 2002; Valko et al. 2007).

Glucose toxicity, leading to chronic hyperglycaemia in diabetes, involves oxidative stress as a major regulatory mechanism for inducing progressive β-cell loss and various metabolic complications such as insulin resistance. Pancreatic islets are very sensitive to oxidative stress since they express very low amount of anti-oxidant enzymes, like superoxide dismutases (SOD-1, SOD-2), catalase and glutathione peroxidise (GPx) (Grankvist et al. 1981; Tiedge et al. 1997). GSH is the major intrinsic antioxidant in cells. It has been reported, that long-term exposure to high glucose concentration decreases GSH level (Catherwood et al. 2002). Many clinical studies have documented chronic oxidative stress in type 2 diabetes generating excessive ROS causing islet cell death (Sakuraba et al. 2002; Padgett et al. 2013).

In type-1 diabetes, β-cell mass is strictly reduced by 70–80% at an early stage, unlike type-2 diabetes where, the same occurs at relatively slower and gradual pace. However, because of the variable degree of insults and absence of detectable β-cell necrosis, it is suggested that β-cell loss occurs slowly over years (Kloppel et al. 1985). These histopathological findings are in line with the progressive decline in first-phase insulin secretion in antibody-positive individuals, long before the development of overt diabetes (Srikanta et al. 1983). Further, it has also been later shown that β-cell apoptosis causes a

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gradual β-cell depletion in rodent models of type-2 diabetes. Activated macrophages secrete cytokines which are responsible for β-cell apoptosis, probably mediated by three main pathways – namely JNK, ER stress, and liberation of pro-apoptotic proteins from the mitochondria (Kutlu et al. 2003).

In type 2 diabetic subjects, initial histopathologic studies suggested β-cell loss by 25–50% (Kloppel et al. 1985; Clark et al. 1988), but this was debated by others (Guiot et al. 2001). A few earlier studies which match diabetic patients and control subjects for BMI, showed a significant reduction in β-cell mass and around a three-fold increase in β-cell apoptosis (Sakuraba et al. 2002; Butler et al. 2003). These observations suggest that β-cell mass is decreased in type 2 diabetes, secondary to increased rates of β-cell apoptosis, but it remains unclear whether this explains the observed functional loss (Kahn 2003). β-Cell apoptosis may thus be a common feature of both type 1and type 2 diabetes.

Natural flavonoids are emerging as potent therapeutic drugs for free radical-mediated diseases. *Enicostemma littorale* (EL), a herb of the Gentianaceae family, contains diversified phytochemicals: some of the important constituents of the plant include betulin, a triterpenoid sapogenin, swertiamarin, a secoiridoid glycoside, monoterpene alkaloids like enicoflavine, swertisin, a flavonoid and gentiocrucine (Saranya et al. 2013). Also, as described by Ambikapathy et al. (2011), some of the major components of the EL MeOH ext. are laminaribiitol (79.93%), 12-hydroxy-9-octadecenoic acid (9.546%), myricetin (4.7519), 3.3-methylenebis (4-hydroxycoumarin) (2.811). Cabechin (2.002) and the total phenolic content have been found to be approximately 5–6 mg gallic acid equivalents (GAE)/g DW (dry weight) (Abirami & Gomathinayagam 2013). Extract of the plant and its major phytochemical swertiamarin have been reported as antioxidant candidates (Vaijanathappa & Badami 2009). EL is known to have good antioxidant activity in diabetic rats (Maroo et al. 2003) as well as in newly diagnosed NIDDM patients (Vasu et al. 2003).

As mentioned above, our previous lab studies demonstrated a series of beneficial effects of EL on diabetic patients as well as in diabetic rat model. Hence, in this current study we wanted to further explore the cytoprotective potential of EL on oxidative stress-induced DNA damage and apoptosis in Islets of Langerhans.

Materials and methods

Animals

Adult virgin male rats of Charles-Foster strain weighing 250–300 g aged 6–8 weeks were kept under controlled conditions of light and temperature. They had access to food and water *ad libitum*. The experimental studies were performed after the approval from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Department of biochemistry, The M. S. University of Baroda and all applicable guidelines for the care and use of animals were followed.

Plant material and preparation of methanolic extract

Whole dried plant was procured from Saurashtra district in Gujarat state, India, in August 2013 after authentication from taxonomist Prof. S. D. Sabnis with voucher specimen number Oza 51, 51 (a) present in the Herbarium, Department of Botany, The M.S University of Baroda, Vadodara, Gujarat, India and all the applicable guidelines for the plant studies were followed. Whole dried plant was used for extract preparation. Plant was cut into small pieces and extracted with petroleum ether for 12 h in a Soxhlet apparatus. Residues were again extracted with methanol for 24 h. After the extraction, methanol was recovered by distillation and remaining traces of methanol were completely removed by keeping methanol extract at 60 °C for 4 days (Maroo et al. 2003; Vasu et al. 2003).

Chemicals

All chemicals and culture media used in this study were purchased from Sigma Aldrich, Bangalore, India. The details of the antibodies used are given in Table 1. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas/Thermo Fisher Scientific, Bangalore, India.

Islet isolation and treatment

Islets were isolated from healthy non-diabetic rats by the method of Xia and Laychock (1993), and then subjected to purification. Here, maintaining sterile conditions, islets were first excised out and thoroughly minced. The minced tissue was collected and given a wash with cold phosphate buffer saline (PBS). The tissue was introduced into a 5 ml Hank's balanced salt solution (HBSS) containing 5 mg collagenase type V. Digestion was carried out for 15 min in a shaking water bath at 37 °C. The digestate was diluted with cold PBS. The digested tissue was allowed to settle and washed thrice. Islets in the digestate were handpicked using Pasteur pipette under dissecting microscope (approx. 1000 islets/group). Purified islets were incubated with cumene H_2O_2 at a dose of 50 µM for 30 min then centrifuged at 400*g* for 10 min, washed twice with PBS

Table 1. List of antibodies along with other details.

Sl. No.	Name of Antibody	Company	Mono/Polyclonal	Mol. Wt.(kDa)	Source	Dilution for Western Blotting/ Immunocytochemistry*
1	Caspase-3	Thermo Pierce	Polyclonal	32& 17,12	Rabbit	1:500
2	PARP	CST	Monoclonal	116(Full length) & 89(cleaved)	Rabbit	1:1000
3	Phospho-P-38 MAPK	CST	Monoclonal	43	Mouse	1:1000
4	MafA	Sigma	Monoclonal	40	Rabbit	1:1000
5	TNF-α	CST	Polyclonal	25(precursor) and 17(mature)	Rabbit	1:1000
6	β-Actin	BD	Monoclonal	42	Mouse	1:5000
7	Annexin V-FITC	BD	–	–	–	1:50*

and used for measurement of different parameters. Another group of islets was pre-incubated with 2 mg/ml EL MeOH ext. for 2 h, washed with PBS and then subjected to 50 µM H_2O_2 exposure. This treatment of EL MeOH ext. and H_2O_2 was uniformly maintained throughout the study. *In vitro* dose (five doses) and time (four time points) dependent study was carried out with extract. The viability of the islets was checked by trypan blue dye exclusion test using 0.4% (W/V) trypan blue (Shewade et al. 2001). Blue stained islets were scored as non-viable and the unstained were scored as viable islets.

Assessment of cell death by PS/PI dual staining

One hundred islets of Langerhans were pelleted and washed twice with PBS. To differentiate between apoptotic and necrotic cell death, dual staining with PS (Annexin V-FITC)/PI (propidium iodide) was performed as described by Rieger et al. (2011). Fluorescence was monitored at 40× magnification using Confocal microscope (LSM, 710 Zeiss, Bangalore, India). The relative islet fluorescence intensity was measured by Image J software (NIH, Bethesda, MD) and then plotted.

Assessment of intracellular oxidative stress using DCFDA (2′,7′–dichlorofluoresceindiacetate) dye

One hundred islets were handpicked and pelleted by centrifuging at 400*g* for 3 min in a 1.5 mL micro centrifuge tube. Media was aspirated and these islets were washed with PBS and re-suspended in the 990 µL PBS along with 10 µL of 10 µg/mL DCFDA dye for 20 min at room temperature. To this suspension 1 µL of 1 µg/mL DAPI stain was added and incubated further for 10 min. Islets were spun down at 400*g* for 3 min and washed twice with PBS. Finally, islets were re-suspended in 50 µL of PBS and mounted and a glass slide and observed under a phase contrast fluorescent microscope. The relative islet fluorescence intensity was measured by Image J software and then plotted as previously reported by Burgess et al. (2010).

Assessment of islet viability using FDA (fluorescein diacetate)/PI staining

One hundred islets were stained with FDA/PI similarly as described in protocol for DCFDA staining. FDA and PI were used at a final concentration of 0.5 and 2 µg/mL respectively.

Comet assay

DNA single strand breaks were measured using the alkaline comet assay. Glass microscope slides were frosted with 1% normal melting point Agarose (type I-A) prepared in deionized water. Islet of Langerhans were re-suspended in 400 µL of 0.8% low melting point Agarose (type VII A) in PBS at 37 °C and pipetted onto a frosted microscope slide pre-coated with 100 µL of 1% normal melting point Agarose. Slides with layers of cells in agarose were incubated at 4 °C for 10 min and then immersed in lysis solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, 1% Triton X-100, pH 10) for 1 h to remove cellular membranes. After lysis, slides were placed in a horizontal electrophoresis tank containing electrophoretic solution (1 mmol/L Na_2EDTA, 300 mmol/L NaOH, pH 13) at 40 °C for 40 min. (DNA unwinding). Electrophoresis was performed in the same solution at 25 V, 300 mA, 40 °C for 30 min. The slides were washed three-times with neutralizing buffer (0.4 mmol/L Tris, pH 7.5) for 5 min at 40 °C before staining with 4, 6-diamidine-2-phenylinole dihydrochloride-DAPI (1 µg/mL).

Biochemical analysis

Reduced glutathione level was determined using 5,5′-dithio-bis (2-nitrobenzoic) acid (DTNB) which form a highly yellow colored compound developed spectrophotometrically at 412 nm (Beutler & Gelbart 1985). Lipid peroxidation was estimated as described by Ohkawa, where malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS) (Ohkawa et al. 1979). TBARS gives a characteristic pink color which can be

measured colorimetrically at 532 nm. The superoxide dismutase activity was measured by inhibiting pyrogallol auto-oxidation under standard assay conditions, the reaction was measured at 420 nm spectrophotometrically (Marklund & Marklund 1974). Catalase decomposes H_2O_2, which was measured at 240 nm (Aebi 1984). Glutathione peroxidase (GPx) was measured as discussed by Hafeman et al. (1974). Estimation of caspase-3 activity was using the instruction given in the kit (Sigma, Bangalore, India, # CASP-3-C).

Immunoblotting

Islets were collected by centrifugation and suspended in lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) containing 1× proteinase inhibitors (Sigma, Bangalore, India), incubated on ice for 30 min. After centrifugation at 16 000*g* for 15 min at 4 °C, the supernatant was collected and kept at −80 °C for future use. Total protein content was quantified using Bradford assay (Bio-Rad Bradford Solution, Haryana, India). The protein (20 µg) was loaded on a 10% polyacrylamide gel and then electrophoretically transferred onto a Hybond Nitrocellulose membrane (GE Healthcare, Bangalore, India). The membrane was then incubated for 1 h at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further incubated overnight with the primary antibody at 4 °C (Table 1). Membrane was then washed four times 15 min each with TBST and incubated with HRP-conjugated secondary antibody for 1 h (Table 1). Finally, membrane was developed and visualized with Enhanced Chemiluminescence Western Blotting detection system (Millipore Inc., Bangalore, India).

Results

Islet isolation and viability

Rat islets were isolated using collagenase V digestion and handpicked for uniform size architecture. Islets were stained with dithizone stain to confirm the purity, and before each experiment, isolated islets were more than 99% viable, which was confirmed using Trypan blue staining, ensuring live healthy islets to study apoptosis and DNA damage (Figure 1a).

Standardization of effective dose and time of EL MeOH extract for cytoprotective activity

Dose and time-dependent experiment were performed in order to standardize effective dose and time of EL MeOH extract for protection against H_2O_2 induced loss of islets viability. In order to assess the cytoprotective effect of EL on Islet of Langerhans against the oxidative stress, Trypan blue dye exclusion test was performed. Islets were pre-incubated with EL MeOH ext. at 0.25, 0.5, 1, 2 and 4 mg/mL of assay volume for 0.5, 1.0, 2.0 and 4.0 h each. Results demonstrated partial protection against H_2O_2 induced toxicity in a dose and time dependent manner. We observed that pre-incubation before H_2O_2 treatment of 0.25 mg/mL EL MeOH ext. till 1 h had no protective effect. However, 2 mg/mL EL MeOH ext. for 2 h gave optimum cell viability of 58 ± 1.8% when compared to H_2O_2 treated islets which do not show any cell viability (Figure 1b).

FDA/PI staining for viability assessment

We performed islet viability both using FDA/PI staining. FDA stains live cells with green fluorescence, while PI stains dead cells giving red fluorescence. Freshly isolated rodent islets when stained for FDA/PI showed maximum viability by green fluorescent FDA staining while PI stained dead cells (Figure 2a). Further, when these islets were exposed to 50 µM H_2O_2, they showed enormous islet death as demonstrated by significant increase in the PI red fluorescence and decrease FDA green fluorescence intensities. While in other case, islets when pre-incubated with EL MeOH extract, we found enhanced protection to cytotoxicity in islets by 50 µM H_2O_2 and significantly fewer number of PI stained dead cells (Figure 2a and b). These results suggested that EL MeOH extract pretreatment provides enhanced protection against oxidative stress induced cell death.

DCFDA staining for intra-cellular ROS generation

DCFDA is a measure for estimating intra-cellular ROS generation. Mild exposure of islets to H_2O_2 show increased level of intra-cellular oxidative stress (Figure 2c), when quantified was found to be approximately 4-fold higher compared to control islets (Figure 2d). Also, islets first pre-incubated with EL MeOH extract and later exposed to H_2O_2 showed highly reduced ROS generation within cells with low green fluorescent intensity, evidently suggesting normalized intracellular ROS stress. Thus, ROS estimation clearly indicates preventive potential of EL MeOH extract to islets rendering cytoprotection by lowering ROS generation similar to control islets.

Phosphotidylserine and propidium iodide staining in islets for live and dead cells

We further looked for apoptotic and necrotic cells both qualitatively and quantitatively by PS/PI dual staining.

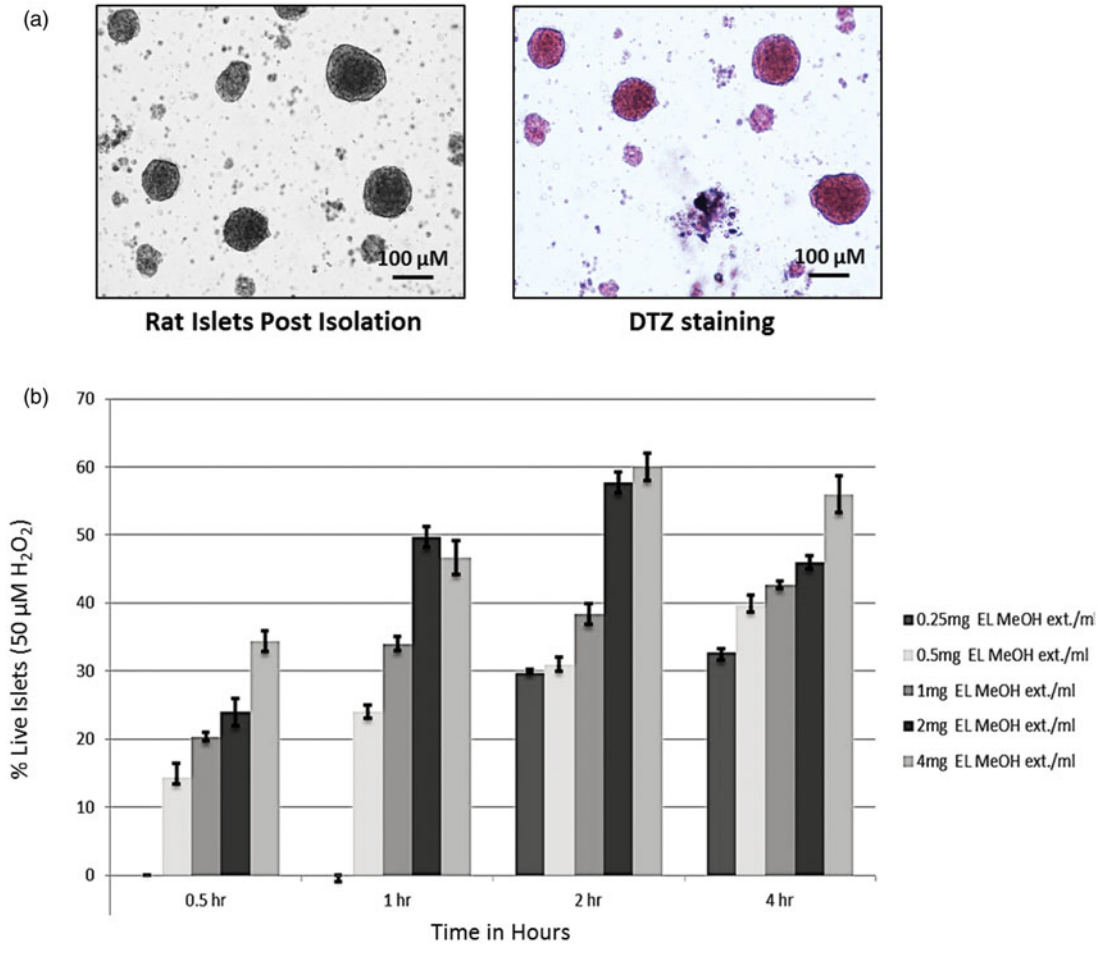

Figure 1. Trypan blue dye exclusion assay of isolated rat islets: (a) Isolated islets and DTZ staining. (b) Trypan blue dye exclusion assay to identify the pretreatment dose and time of incubation for EL MeOH ext. in a time dependent and dose dependent study assessing its maximum protective effect against 50 µM H_2O_2 induced cell death ($n = 3$).

Freshly isolated islets after isolation did not show PS staining with fairly low apoptotic nuclei. On the other hand, Islet when exposed to H_2O_2 alone, showed significant increase in the apoptotic nuclei (red) on exposure with 50 µM H_2O_2 for 0.5 h with Annexin-V binding to phosphotidylserine (green). However, pre-incubating islets with 2 mg/mL of EL MeOH extract significantly inhibited H_2O_2-induced apoptosis (Figure 3a). It was observed that both Annexin-V-FITC and PI fluorescent intensities when quantified demonstrated significant reduction after EL MeOH ext. pre-treatment (Figure 3b).

Comet assay for oxidative stress induced DNA damage

Damage to cellular DNA integrity, induced by H_2O_2 exposure was detected using an alkaline comet assay experiment. Acute exposure of islet cells to H_2O_2 facilitated increase in DNA damage which was observed by comet tail length. Control islets did not have stress and showed no tailing. Islets treated with 50 µM H_2O_2 demonstrate comet tailing suggested evident DNA damage in response to oxidative stress. However, islets pre-incubated with EL MeOH extract and then exposed to 50 µM H_2O_2 showed reduced comet tail length, indicating a protective effect over H_2O_2 induced DNA damage, hence cytoprotection (Figure 3c).

Immunoblotting and quantification of cell death markers due to oxidative stress

Oxidative stress results in DNA damage and activates DNA repair enzymes. This increase in DNA damage leads to PARP-1 activation causing depletion of cellular energy supply which drives the cell into apoptosis *via* activating caspases, an apoptotic executor. caspase-3 cleaves PARP-1 and abolishes its enzymatic activity that abates further energy depletion thus preventing cells from going into necrosis (Luo & Kraus 2012). Also, Oxidative stress increases the expression of inflammatory cytokines such as TNF-α and stress

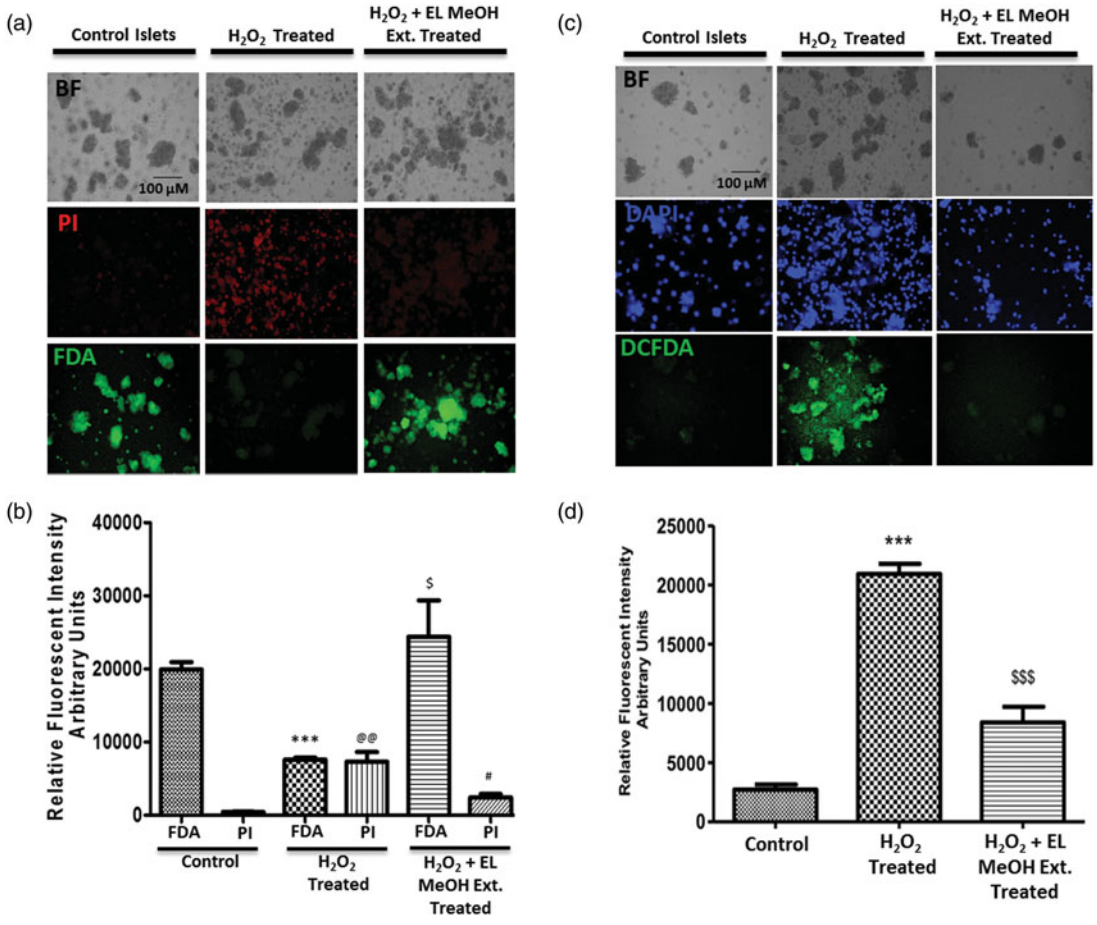

Figure 2. FDA/PI and DCFDA Staining: (a) Qualitative assessment of islet viability when exposed to 50 µM H_2O_2, when pre-incubated with EL extract compared to the control group. (b) Comparative fluorescent intensity plot for FDA/PI staining (***$p \leq 0.001$ versus Control FDA; @@$p \leq 0.01$ versus Control PI; \$$p \leq 0.05$ versus H_2O_2 treated FDA; #$p \leq 0.05$ versus H_2O_2-treated PI). DCFDA staining representing the amount of ROS generation. (c) Comparative qualitative assessment of the DCFDA staining between the control, 50 µM H_2O_2 and the EL extract-treated groups. Here, the DCFDA gives green fluorescence in the presence of ROS in direct proportion. Hence, this figure indicates the presence of ROS, which is higher in the H_2O_2-treated group compared to EL treated and (d) Quantitative graphical representation for the same (***$p \leq 0.001$ versus Control group; \$\$\$$p \leq 0.001$ versus H_2O_2-treated group) ($n = 3$).

kinases like Phospho-p38 MAP kinases (Cnop et al. 2005). Herein, we attempted to analyze various cell death and stress markers by immunoblotting. Islets treated with and without hydrogen peroxide and pre-incubated with islets were assessed on gels and observed for markers like PARP-1, caspase3, TNF-α and p-38 MapK. In our experiments, we observed a relative increase in procaspase-3 and PARP-1 levels upon oxidative stress induction along with activated caspase-3 and cleaved PARP-1 levels in 50 µM H_2O_2 treated group. However, islets pre-incubated with EL MeOH extract demonstrated basal levels of PARP-1 and pro-caspase-3 (Figure 4a). Also, densitometric quantification showed significant reduction in both PARP-1 cleavage and caspase3 activation (Figure 4b and c). This pretreatment rescued the cells from DNA damage and death by oxidative stress. We further looked for MafA, a specific marker for the β cells, which did not change within the groups. This indicated uniform presence of β cells within the experimental islet groups (Figure 4a and d). We then looked for p-38 MapK phosphorylation which is a stress kinase and expressed in response to external or internal oxidative and other stress factors. Control islets did not show any rise in p-38 phosphorylation, whereas islets exposed to H_2O_2 demonstrated high p-38 phosphorylation due to ROS generation demonstrating ER stress in islet cells and thus cytotoxicity. Moreover, when islets were pre-incubated with EL MeOH extract, we found complete rescue of cells from this cytotoxicity by reverting p-38 phosphorylation back to basal levels as present in control islets (Figure 4a and e). TNF-α is a cytokine that plays a central role in islet inflammatory response pathway. Cytokines induce cell death similar to oxidative stress induced mechanism. Hence, it becomes evident to look for marker of cytokines mediated cell death induction. Therefore, in our case, we observed

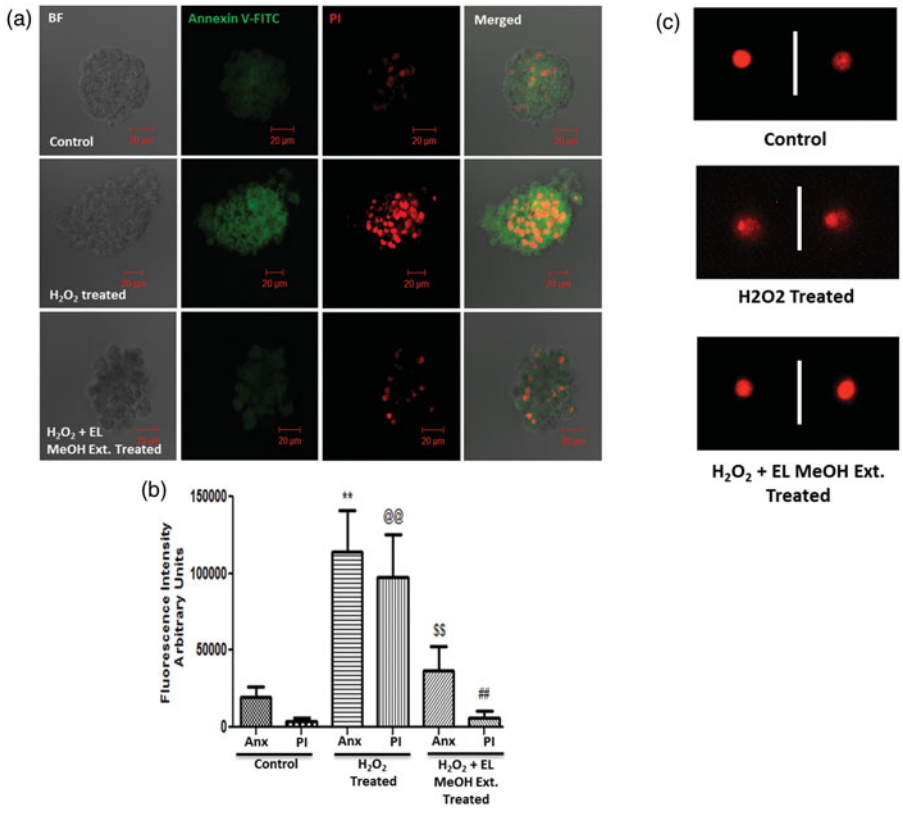

Figure 3. Cell death determined by Annexin V-FITC (PS)/PI staining and DNA damage by comet assay: (a) H_2O_2-treated group demonstrated significant increase in staining for PS and PI from the control group indicating cell death. However, there was no difference in staining of PS and PI in the EL pre-treated group and the control group but a significant reduction in stain uptake between the H_2O_2-treated and EL pre-treated group, which suggested that pre-incubation with EL protects the islets from undergoing oxidative stress induced cell death, which was quantified and depicted (b) (**$p \leq 0.01$ versus Control Annexin V; @@$p \leq 0.01$ versus Control PI; $$$p \leq 0.01$ versus H_2O_2 treated Annexin V; ##$p \leq 0.01$ versus H_2O_2-treated PI). (c) Comet Assay where the tailing is more pronounced in the case of H_2O_2 treated group suggesting DNA fragmentation, whereas the tailing is not seen in the case of EL-treated group giving a direct evidence of effective protection against DNA damage ($n = 3$).

TNF-α activation in response to H_2O_2 induced cytotoxicity. As expected, control islets did show low levels of TNF-α precursor but no activation. Islets exposed to H_2O_2 showed a steep rise in TNF-α activation and precursor both, suggesting cytotoxicity via cytokine mediated cell death. However, islets pre-incubated with EL MeOH extract demonstrated rescue of cell from cytokine mediated cell death. EL treatment significantly drops TNF-α precursor level and its activation, demonstrating its cytoprotective effect on β-cells (Figure 4a and f).

Antioxidant enzymes activity in H_2O_2 exposed and EL MeOH treated islets

Activated caspase-3 is a gold standard marker for cell death, hence caspase-3 enzyme activity was investigated in control, cumene H_2O_2 exposed and pre-incubated islets. We observed that pre-incubating islets with EL MeOH extract itself is sufficient to significantly reduce caspase-3 activity by 42% (Figure 5a). While looking on other antioxidant enzymes, 50 µM H_2O_2 exposed islets showed significantly increased lipid peroxidation levels (101%) but decreased GSH (75%), SOD (43%), catalase (53%) and GPx (44%) activities. However, pre-incubation with EL MeOH extract for 2 hours showed decreased lipid peroxidation (58%) whereas increased GSH content (68%), SOD (55%), catalase (51%) and GPx activities (44%) were observed respectively (Figure 5b–f).

Discussion

Diabetes mellitus is characterized by hyperglycaemia due to excessive ROS generation within beta cells (Bonnefont-Rousselot 2002). Islets are known to have intrinsically very-low levels of antioxidant enzymes expression and activities (Lenzen et al. 1996; Tiedge et al. 1997). Hence, islets are at higher risk for ROS induced oxidative damage compared to other tissues. Many reports have shown that transient exposure of

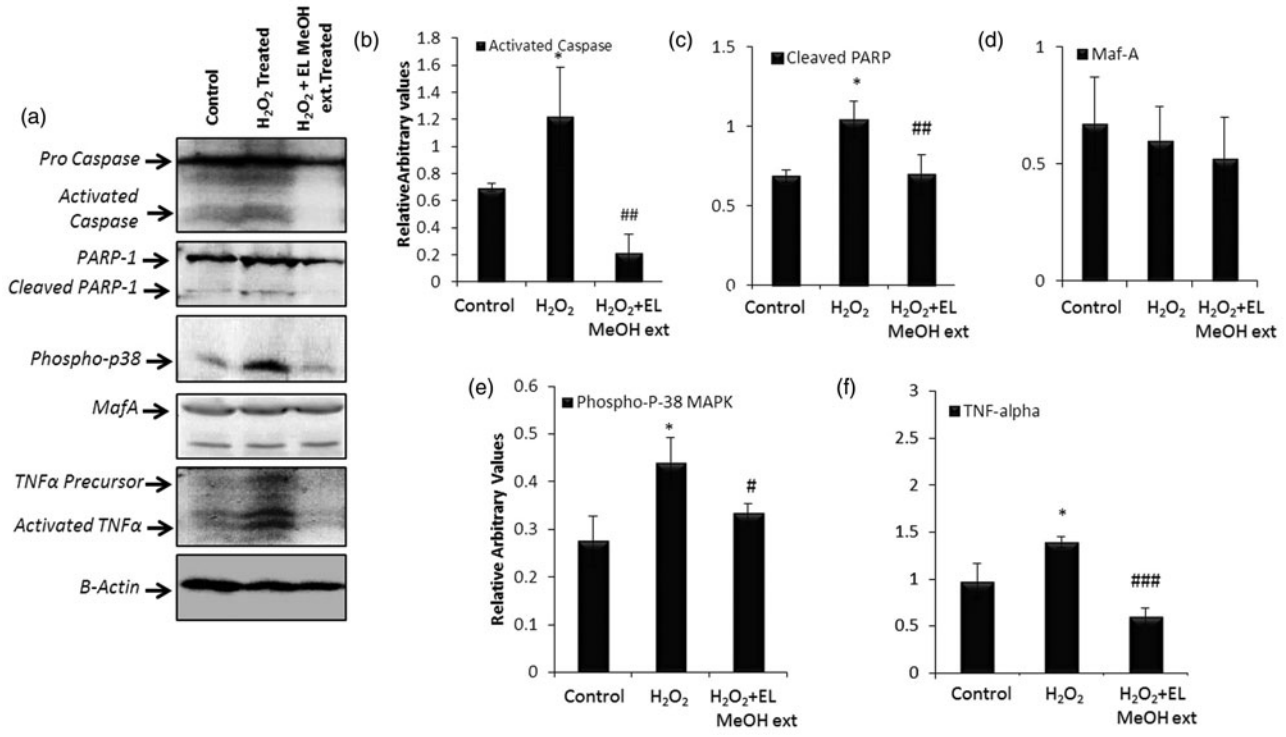

Figure 4. Immunoblotting assay: (a) Western blot analysis for the following proteins to evaluate the protective effect of EL extract against 50 μM H_2O_2 induced apoptosis, Caspase-3, PARP-1, TNF-α, MafA, P-P38 MAPK and β-actin as endogenous control. Further, the protein expression profile is analyzed densitometrically. (b, c, e and f) Densitometry plots of activated caspase-3, cleaved PARP-1, phospho p-38 MAPK and TNF-α respectively where EL pretreatment significantly decrease there expression level after H_2O_2 treatment back to that of the control expression levels. (d) Densitometry plot for MafA which remain unchanged as discussed above (* indicates versus Control and # indicates versus H_2O_2-treated group; */#$p \leq 0.05$; **/##$p \leq 0.01$ and ***/###$p \leq 0.001$) ($n = 3$).

H_2O_2 is sufficient to cause significant damage to beta cells (Tang & Zhang 2000). In pancreatic islets, antioxidant enzymes CAT, SOD and GPx are expressed at fairly low levels (Robertson et al. 2003). The high expression levels of catalase and SOD in insulin secreting cells through genetic engineering provide protection against the toxicity of reactive oxygen species (Tiedge et al. 1998; Bottino et al. 2002). Herbs such as *Curcuma longa* have been shown to abate oxidative stress (Quiles et al. 2002) while its compound curcumin has been used to reduce ROS generation and increase insulin secretion from islets (Amoli et al. 2006; Kanitkar & Bhonde 2008); Puerarin, the main isoflavone glycoside found in the Chinese herb, Puerariae Lobatae Radix (*Pueraria lobata* (Willd) Ohwi), has been shown to have antioxidant activities such as increasing CAT and SOD activity in rat islets and to protect them from toxic effects of H_2O_2 (Xiong et al. 2006). Green tea (*Camellia sinensis*) extract when supplemented to streptozotocin induced diabetic rats also decreased serum glucose and increased serum and hepatic total antioxidant capacity (Haidari et al. 2013). Similarly, studies with other herbal extracts in streptozotocin-treated diabetic rat models have demonstrated their protective potential against oxidative stress induced damage by elevating antioxidant activity (Sarkhail et al. 2007; Stephen Irudayaraj et al. 2012; Nain et al. 2012). In another such study, it was demonstrated that *S. lavandulifolia* extract possessed marked anti-oxidative stress activity and it can be useful as a supplement in the management of diseases related to oxidative stress in humans (Rahzani et al. 2013). Also, hypoxia which occurs during the islet transplantation procedure initiates a cascade of biochemical reactions which results in the production of ROS causing oxidative stress leading to necrosis and apoptosis via intracellular pathways. Hence, incorporation of effective antioxidant supplementation in such procedures can enhance the chances of islet graft survival (Ramkumar et al. 2013).

Our present study coincides with many other earlier reports. *Enicostemma littorale*, a perennial herb, has been used for therapy of diabetes mellitus in India as a folk medicine (Saranya et al. 2013). Very recently we also demonstrated that EL MeOH extract possess potential islet neogenic activity from tissue specific stem/progenitor cells, which was later confirmed by identifying the active ingredient swertisin for this islet neogenic potential in a another recent report from us (Dadheech et al. 2010, 2013). There are also other reports where decrease in insulin resistance, normalization of fat and carbohydrate metabolism by EL were observed in both

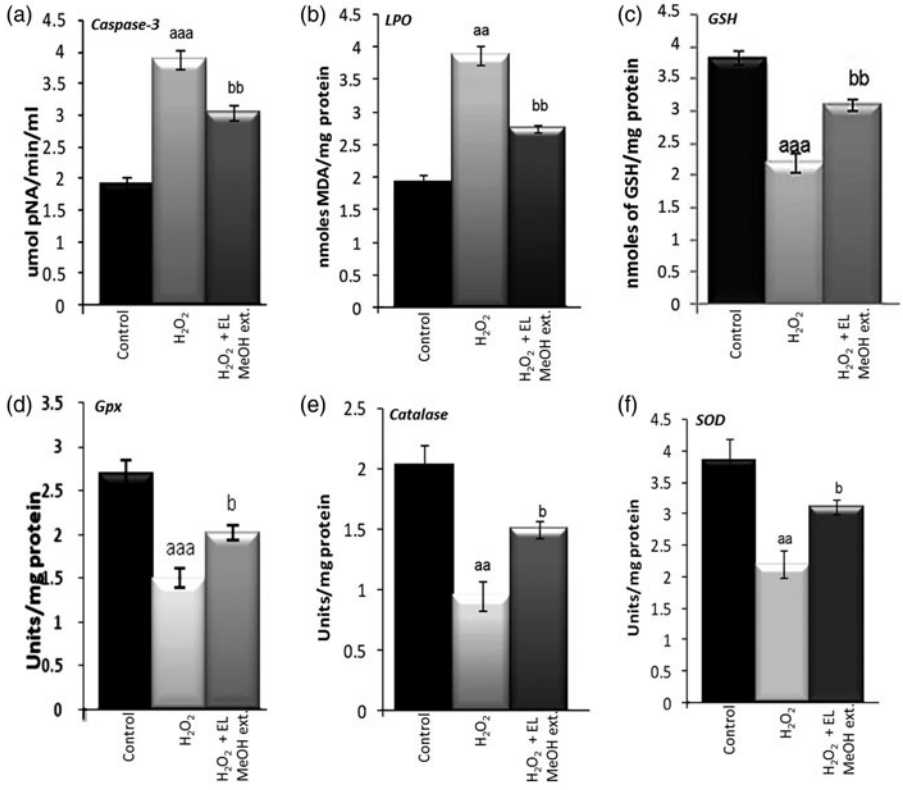

Figure 5. Effect of EL on caspase-3 activity and the antioxidant enzyme activities viz. reduced glutathione, lipid peroxidation, glutathione peroxidase, catalase and superoxide dismutase in isolated islets on 50 μM H_2O_2 exposure and EL MeOH ext. pretreatment: (a) Caspase-3 activity which significantly increases on exposure to H_2O_2 and reduces significantly when pretreated with EL. (b) Similar results with LPO is shown. (c, d, e and f) Representation of antioxidant enzyme activities of GSH, Gpx, catalase and SOD respectively. Their activities significantly decrease in response to H_2O_2 induced oxidative stress but gets rescued with pretreatment with EL back to control levels (a indicates versus Control and b indicates versus H_2O_2 treated group; a/b$p \leq 0.05$; aa/bb$p \leq 0.01$ and aaa/bbb$p \leq 0.001$) $n = 3$).

experimental cellular and rodent models (Maroo et al. 2002; Vasu et al. 2003; Patel et al 2013). Further, decreased oxidative stress by amelioration of antioxidant status in diabetic animals has also been observed (Maroo et al. 2003). EL has also been shown to possess hypolipidemic, hepato-protective, anti-edematogenic and free radical scavenging properties (Vasu et al. 2005; Vaijanathappa & Badami 2009). In the present study, we highlight novel cytoprotective and anti-apoptotic activity of EL just with pre-incubation of islets with 2 mg/mL EL MeOH extract for 2 h. This report imparts concrete evidence for significant improvement of GSH content, SOD, CAT and GPx activities in just 30 min on pre-incubation of islets before cytotoxicity. These antioxidant enzyme status and improved functionality suggests that the EL alone can strengthen the ability to combat oxidative stress and impart cytoprotection to cells against oxidative stress induced damage.

Further, caspase-3, the apoptotic executor can be activated by H_2O_2 (Turner et al. 1998). Also, PARP-1, DNA repair enzyme is activated in response to DNA damage caused by oxidative stress. Mild exposure to oxidative stress causes DNA damage and so, activation of PARP-1, leading to excessive energy consumption hence, apoptosis. Excessive depletion of energy further leads to caspase-3 activation and PARP cleavage, inhibiting PARP over activation. This allows the cells to enter apoptosis, thus preventing necrotic onset (Luo & Kraus 2012). In this study, we could show that H_2O_2-treated islets showed increased activity and expression of activated caspase-3 and PARP-1 cleavage indicating more apoptosis, which gets rescued very efficiently by just pre-incubating islets with EL MeOH extract. This was confirmed by significant reduction in the levels of activated caspase-3 and cleaved PARP-1. It has been reported that hydrogen-peroxide-associated ROS-mediated DNA damage takes place via direct attack on chromosome or mitochondrial pathways, leading to necrosis or apoptosis (Valko et al. 2007). Oxidative stress-induced cellular death can be prevented by blocking DNA damage. Our results from this study suggest that the EL MeOH extract protects H_2O_2 induced cells from apoptosis by efficiently blocking DNA damage.

Many reports have very well documented p38MapK, a stress kinase, affecting variety of intracellular responses,

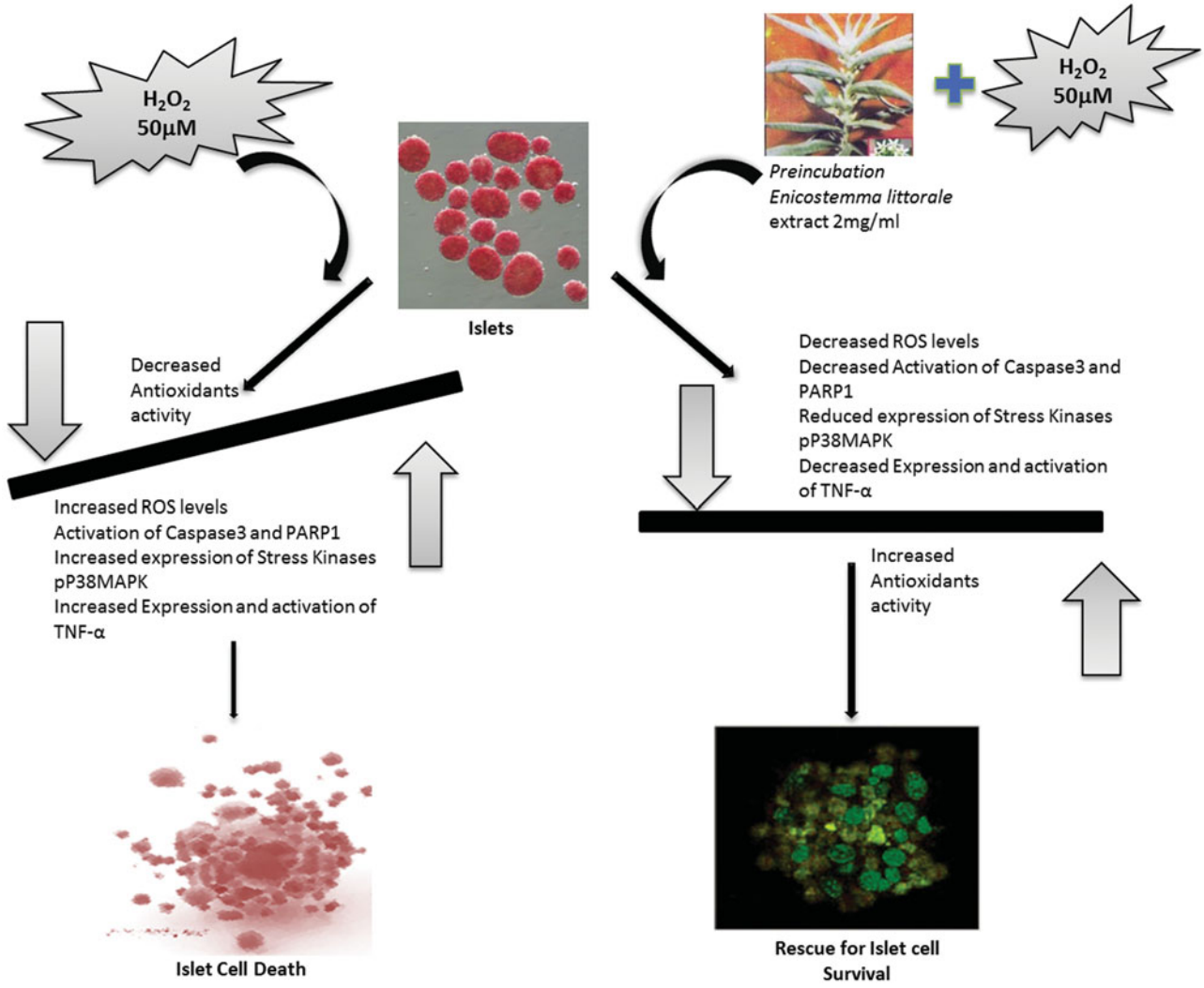

Figure 6. Graphical summary: The figure represents a graphical summary of the entire manuscript exploring the novel protective effect of EL MeOH ext. against H_2O_2-induced oxidative stress in the islets of Langerhans.

with well recognized roles in inflammation and cell death (Turner et al. 1998). Also, p-38 phosphorylation is found to be a standard measure to analyze ER stress in beta cells, leading to beta cell death. In this present study, phosphorylation of p38MapK was observed in the H_2O_2 treated islets, which reverts to normal basal levels with just pre-incubating islets with EL MeOH extract. Similar phenomenon occurs in the case of diabetes, where glucolipotoxicity causes ROS generation. Hence, increased p38MapK signaling has been described in both forms of diabetes, and is associated with late complications, such as ROS-mediated neuropathy and nephropathy (Coulthard et al. 2009). There is a direct correlation between inflammatory cytokines and pathogenesis of diabetes mellitus (Cnop et al. 2005). TNF-α is a known as potent modulator of glucose intolerance and insulin resistance in the peripheral tissue. Also, it is believed that macrophages and adipocytes are the primary sites of TNF-α production. Many groups have demonstrated that purified pancreatic islets serve as a potent source of TNF-α activation upon ROS exposure (Leeper-Woodford and Tobin 1997; Tobin et al. 2001). Hence, these reports explain the sharp increase in the TNF-α levels observed in the islets exposed to H_2O_2 in the case as well. Inflammatory cytokines and stress kinases have a profound inter-dependence that modulate and regulate beta cell function (Wellen & Hotamisligil 2005).

Henceforth, in present study, we demonstrated that p38MapK and TNF-α levels in the EL MeOH extract pre-incubated islets show normalized levels similar to control islets. This strongly suggests that EL has the capacity to counteract both inflammatory cytokines and oxidative stress mediated cytotoxicity. The significance of this study lies in the fact that this study is multifactorial right from isolation and purified islets, and usage of EL alone or in combination can provide efficient cytoprotection to islets while isolation procedure, storage

and transplantation against oxidative stress mediated DNA damage and death (Figure 6).

Conclusion

Our experimental observation and data suggest that EL protects islets or beta cells from oxidative stress (H_2O_2)-induced apoptosis by blocking ROS-mediated DNA damage, increasing antioxidants activity, suppressing expression of intra-islet stress kinases and proinflammatory cytokines. Therefore, this study strongly highlights a novel therapeutic agent that imparts protection to the Islets of Langerhans on various levels against oxidative stress.

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Disclosure statement

We wish to confirm that there are no known conflicts of interest associated with this publication.

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STEM CELLS AND DEVELOPMENT
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Basal Expression of Pluripotency-Associated Genes Can Contribute to Stemness Property and Differentiation Potential

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Pluripotency and stemness is believed to be associated with high *Oct-3/4*, *Nanog*, and *Sox-2* (ONS) expression. Similar to embryonic stem cells (ESCs), high ONS expression eventually became the measure of pluripotency in any cell. The threshold expression of ONS genes that underscores pluripotency, stemness, and differentiation potential is still unclear. Therefore, we raised a question as to whether pluripotency and stemness is a function of basal ONS gene expression. To prove this, we carried out a comparative study between basal ONS expressing NIH3T3 cells with pluripotent mouse bone marrow mesenchymal stem cells (mBMSC) and mouse ESC. Our studies on cellular, molecular, and immunological biomarkers between NIH3T3 and mBMSC demonstrated stemness property of undifferentiated NIH3T3 cells that was similar to mBMSC and somewhat close to ESC as well. In vivo teratoma formation with all three germ layer derivatives strengthen the fact that these cells in spite of basal ONS gene expression can differentiate into cells of multiple lineages without any genetic modification. Conclusively, our novel findings suggested that the phenomenon of pluripotency which imparts ability for multilineage cell differentiation is not necessarily a function of high ONS gene expression.

Introduction

Ability to isolate and establish pluripotent or multipotent stem/precursor population is a major objective for the biotechnological industry and clinical translation of regenerative medicine. A major impediment for using stem cells in a clinical setup is poor availability of cells, especially those obtained with noninvasive procedures without raising much ethical issues. These limitations greatly restrict the usage of stem cells in clinics, disabling treatments of many degenerative diseases. This lacuna can be filled if any tissue-specific cells can be verifiably demonstrated to possess pluripotent or multipotent capacity. This may elevate the hope to find a well-suited stem cell-like cell line that can serve as an autologous, noncontroversial, and renewable source for cell therapy without ethical and immunological concerns, which are usually associated with embryonic stem cells (ESCs).

Numerous gene and protein expression criteria have been set for recognizing a cell as pluripotent. Microarray analyses have demonstrated a set of various transcripts that are associated with stemness as in the case of ESCs [1]. Notably, it has been demonstrated by Yamanaka and colleague that the combinations of four major transcription factors, *Oct-3/4*, *Nanog*, *Sox-2*, and *Klf-4*, is indispensable for the cells to maintain pluripotency as in the case of ESCs [2,3]. However, there are some other reports which indicate that expressions of not all the four genes are essential to maintain the stemness. According to NIH and ISSCR guidelines, teratoma formation is one of the major criteria for classifying a cell to be pluripotent [4]. Apart from this, the presence of alkaline phosphatase (ALP) is another reliable property shown for pluripotent cells such as ESCs [1,5].

In principle, a cell that is able to differentiate into cell types of all three germinal layers is considered pluripotent. On the other hand, a multipotent cell can give rise to cells originating from the same germ layer [6]. A classic example of a stem cell with pluripotent and multipotent potential is mesenchymal stem cells (MSC). MSCs can differentiate into various cell types. Depending on isolation procedure and tissue source, both pluripotent and multipotent type of stem cells can be isolated [7]. Mesenchymal cells display fibroblastic cell morphology and express vimentin protein as a

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Research Article

A Small Molecule Swertisin from *Enicostemma littorale* Differentiates NIH3T3 Cells into Islet-Like Clusters and Restores Normoglycemia upon Transplantation in Diabetic Balb/c Mice

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Aim. Stem cell therapy is one of the upcoming therapies for the treatment of diabetes. Discovery of potent differentiating agents is a prerequisite for increasing islet mass. The present study is an attempt to screen the potential of novel small biomolecules for their differentiating property into pancreatic islet cells using NIH3T3, as representative of extra pancreatic stem cells/progenitors. *Methods*. To identify new agents that stimulate islet differentiation, we screened various compounds isolated from *Enicostemma littorale* using NIH3T3 cells and morphological changes were observed. Characterization was performed by semiquantitative RT-PCR, Q-PCR, immunocytochemistry, immunoblotting, and insulin secretion assay for functional response in newly generated islet-like cell clusters (ILCC). Reversal of hyperglycemia was monitored after transplanting ILCC in STZ-induced diabetic mice. *Results*. Among various compounds tested, swertisin, an isolated flavonoid, was the most effective in differentiating NIH3T3 into endocrine cells. Swertisin efficiently changed the morphology of NIH3T3 cells from fibroblastic to round aggregate cell cluster in huge numbers. Dithizone (DTZ) stain primarily confirmed differentiation and gene expression studies signified rapid onset of differentiation signaling cascade in swertisin-induced ILCC. Molecular imaging and immunoblotting further confirmed presence of islet specific proteins. Moreover, glucose induced insulin release (*in vitro*) and decreased fasting blood glucose (FBG) (*in vivo*) in transplanted diabetic BALB/c mice depicted functional maturity of ILCC. Insulin and glucagon expression in excised islet grafts illustrated survival and functional integrity. *Conclusions*. Rapid induction for islet differentiation by swertisin, a novel herbal biomolecule, provides low cost and readily available differentiating agent that can be translated as a therapeutic tool for effective treatment in diabetes.

1. Introduction

Diabetes is a devastating disease, affecting millions of people worldwide. Hyperglycemia is a principal signature of both type 1 diabetes (T1D) and type 2 diabetes (T2D). Reversal of hyperglycemia by exogenous insulin may delay or attenuate but never eliminate the risk for developing secondary complications [1]. Islet transplantation is a modern approach that has become more prevalent in clinics nowadays. It offers internal glucose homeostasis with low surgery risk and reduces complications in diabetic patients. However, islets derived from multiple donors require immunosuppressors. Also inadequate islet supply from cadaveric pancreas has limited the widespread utilization of this approach [2].

Cell-based therapy, principally new islets derived from stem cell differentiation, is a new area of research in diabetes. Recent studies have shown that embryonic stem cells, induced pluripotent stem cells, adult bone marrow mesenchymal stem cells, and many other tissue-specific progenitors have the ability to convert into cell of multiple lineages like blood, liver, lung, skin, cardiac, muscles, and neurons including insulin producing β cells upon appropriate