Research Article

Effect of Quercetin on Beta cell Regeneration

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Abstract

Objective: The aim of the present study was to investigate the role of Quercetin in beta cell regeneration *in vitro* and *in vivo*. **Methods**: The research work was initiated with *in vitro* experiment wherein 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay was performed using the MIN6 cell line. *In vivo* study was performed in Streptozocin-induced diabetic Wistar rats and Quercetin (QE) was administered orally in three doses (25, 50, 100 mg/kg). Body weights, serum insulin, blood glucose were measured. At the end of the study animals were sacrificed and histological examination was carried out which included normal histopathology, immunohistochemistry and 5-Bromo-2'-deoxyuridine (BrdU) cell proliferation assay. **Results**: Streptozocin damaged MIN6 cells showed significantly ($P < 0.001$) higher viabilities after administration of QE (10µg/ml). QE administration significantly ($P < 0.0001$) increased body weights as compared to Diabetic Control (DC) group. Administration of QE (50mg/kg) and QE (100mg/kg) significantly ($P < 0.0001$) decreased fasting blood glucose level and significantly ($P < 0.001$), ($P < 0.0001$) increased serum insulin level as compared to DC group. Pancreatic Insulin secretion significantly ($P < 0.0001$) increased in QE (100mg/kg) group as compared to DC group, also restoration of islets, reduced pancreatic damage with increase in number of β-cells was observed in QE (100mg/kg) group as compared to DC group. The increased number of BrdU positive cells was observed on QE (100mg/kg) administration as compared to DC group. **Conclusion**: The present study thus confirmed beta cell regeneration using QE.

***Keywords*:** Streptozocin, antibody, diabetes, insulin, MIN6

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder, characterized by the presence of persistent hyperglycemia resulting from defects in insulin secretion, insulin action or both. World Health Organization (WHO) indicates that DM is one of the major killers of humans and is affecting 1–5% of the world population (Mohammed et al., 2016). Type 1 diabetes, or insulin-dependent diabetes mellitus (IDDM), is a common pediatric chronic disease, affecting an increasing number of children every year. IDDM occurs due to autoimmune destruction of insulin producing β-cells in the pancreas, resulting in low or no production of insulin, a hormone necessary for survival (Zimmet et al., 2001). Type I diabetes (T1D) patients rely on cumbersome chronic injections of insulin, making the development of alternate durable treatments a priority (Desgraz et al., 2011). Loss of functional beta cells is fundamental in both type 1 and type 2 diabetes (Cnop et al., 2005). Current treatments for diabetes fail to halt the decline in functional β -cell mass; therefore, strategies to prevent β -cell dysfunction and apoptosis are urgently needed (Meier et al., 2005). It is now appreciated that insulin-secreting pancreatic beta-cells have a finite life span and that dying beta-cells are continuously replaced throughout life. Furthermore, insulin-secreting pancreatic beta-cells can further proliferate in response to increasing demand for insulin and after physiological injury. These observations raise the possibility of enhancing the base-line replication of beta-cells as a therapeutic approach for the treatment of patients with type 1 or type 2 diabetes (Cheng et al.,

2015). Much effort has been made to increase β cell mass by stimulating endogenous regeneration of islets. Beta-cell regeneration, therefore, has garnered great interest as an approach to diabetes therapy (Yin et al., 2013). Indeed, β cell regeneration has been shown to occur at a basal rate in normal adult tissues and to increase under conditions of metabolic stress such as pregnancy, obesity, and diabetes (Chen et al., 2004). Search for anti-diabetic agents have been extended to plant-derived products, since fewer side effects have been reported with the use of plants in the treatment of several diseases (El-Kordy and Alshahrani,

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2015). Plants are rich sources of antidiabetic, antihyperlipidemic, and antioxidant agents such as flavonoids, gallotannins, amino acids, and other related polyphenols (Ahmed et al., 2010). Flavonoids have the capacity to inhibit enzymes such as cyclooxygenases and protein kinases involved in cell proliferation and apoptosis (Vinayagam and Xu, 2015). Quercetin (QE) is a well-known flavonoid and a strong antioxidant widely existed in red wine, onions, green tea, apples, berries, caper, tomato and lettuce (Maciel et al., 2013). Quercetin has attracted increasing attention due to its antioxidant, anti-obesity, anti-carcinogenic, antiviral properties (Wang et al., 2016). QE prevents streptozocin-induced oxidative stress and protects β-cell against damage in diabetic rats (Youl et al., 2010). Thus, the present study was undertaken to investigate the role of QE in beta cell regeneration *in vitro* using MIN6 cell line and *in vivo* using STZ-induced diabetic rats.

Materials and methods

Chemicals

Quercetin, Streptozocin, 3,3'-Diaminobenzidine tablets, Monoclonal Anti-Insulin Antibody Produced in Mouse (Pk Of 100 UL) # I2018 SIGMA (Clone K36AC10, ascites fluid) was purchased from Sigma, USA. BrdU immunohistochemistry kit, Goat Anti-Mouse IgG Antibody # AP308P ((H+L) HRP conjugate) was purchased from Merck, USA. 5-Bromo-2'-deoxyuridine, Dulbecco's Modified Eagle's Medium, Fetal bovine serum, DMSO (Dimethyl sulfoxide were purchased from HiMedia, India. All other chemicals utilized were of analytical grade.

In vitro study

MIN6 cell culture

The MIN6 cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 25mM (4.5g/L) glucose supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100µg/ml streptomycin, 100µg/ml L-glutamine and 5µl/l of 2-mercaptoethanol in humidified 5% CO2, 95% air at 37ºC. Medium was changed every 48h and cells were passaged once weekly following detachment using trypsin-EDTA (Nakashima et al., 2009).

MTT Assay

Mouse insulinoma (MIN6) cells were cultured in DMEM. Cells were grown at 37ºC in a 5% CO2 humidified atmosphere (Kang et al., 2013). Cells were seeded at 1×10^5 per well in a 96-well plate for viability assay. The media cultured with the cells was changed and MTT solution (5 mg/ml in PBS) 20 µL was added to each well and the plates were further incubated for another 6 h. Supernatants were then discarded and 150 µL of DMSO was added to the each incubation well and mixed thoroughly to dissolve the dark blue crystal formations. The absorbance at 570 NM (formation of formazan) was recorded with a microplate spectrophotometer (Lei et al., 2012).

Experimental Design

Grouping of MIN6 cells (n = 3 wells)

NC (Normal Control): No treatment

DC (Diabetic Control): STZ exposed cells

DQ10 (Diabetic QE): Cells exposed to STZ + QE (10µg/ml)

DMSO (Diabetic DMSO): Cells exposed to STZ + DMSO

CQ10 (Control QE): MIN6 cells exposed to QE (10µg/ml)

STZ-induced MIN6 cell-injury model was adopted to investigate the regenerative effect of QE on β cells. Aliquots of 1×10^5 MIN6 cells were transferred into the wells of 96-well cell culture plate. After 48 h, STZ solution (final concentration 6 mM) was added to the each well of 96-well-plates and the cells were exposed to STZ for 24 h or were kept untreated as normal control. STZ treated cells were further exposed to QE (dissolved in DMSO), with final concentration of 10µg/ml for 24 h or kept untreated as diabetic control. MTT assay as mentioned above was performed at the end of the *in vitro* study to determine cell viability (Kang et al., 2013; Lei et al., 2012).

In vivo study

Animals

Sixty adult male Wistar rats with body weights of 150–180 g each, were used for the study. The animals were fed on a standard laboratory, food and water ad libitum and were kept under standard conditions of temperature and humidity. The study was conducted in accordance with the CPCSEA guidelines for animal experimentation and was approved by the Institutional Animal Ethics Committee (IAEC -101-15/2016).

Grouping of animals and drug treatment

Rats were classified into 5 groups of 10 rats each

Normal control (NC): Received no treatment.

Diabetic Control (DC): This group included STZ-induced diabetic animals.

DQ25: Diabetic animals received Quercetin 25 mg/kg.

DQ50: Diabetic animals received Quercetin 50 mg/kg.

DQ100: Diabetic animals received Quercetin 100 mg/kg.

QE, was freshly prepared in 25% ethanol and was administered once daily for a month, by gavage. The dose of QE was adjusted weekly, according to the body weight of the rats. Individual drug dosing was done and the volume of ethanol was adjusted according to the proportional 0.001 ml per 1 g of body weight (Vessal et al., 2003).

Induction of diabetes

Type 1 diabetes mellitus was induced by a single intraperitoneal injection of STZ. STZ was dissolved in 0.1 M sodium citrate buffer (pH 4.5) and injected at a dosage of 55 mg/kg. To overcome the expected hypoglycemia, the animals were allowed to drink 5% glucose solution overnight (Mohammed et al., 2015a). Weight was checked before and after STZ injection. One week after STZ injection, diabetes was confirmed by measuring fasting blood glucose levels from the tail vein using Accu-Chek glucometer (Roche; Stuttgart, Germany) (Chandran

et al., 2016). Only animals with fasting plasma glucose level above 200 mg/dl were chosen for the experiment. Treatment of diabetic rats with Quercetin was started 1 week post STZ injection (Sato et al., 2014).

Determination of body weight

Body weights of all rats were measured at the beginning and the end of the study.

Determination of fasting blood glucose

The rats were fasted overnight and blood samples were taken from the tail vein. The blood glucose was determined using glucometer (Rifaai et al., 2012).

Determination of serum insulin

At the end of the study blood was drawn by puncturing retro-orbital plexus under diethyl ether anaesthesia (Chowtivannakul et al., 2016). The blood samples were centrifuged at 3000 RPM for 15 min and serum from each blood sample was separated (Beck et al., 2015). Serum insulin level was measured by using ELISA method.

Histological examinations

After one month of treatment, rats were sacrificed by cervical dislocation technique, the pancreatic tissues were harvested from the animals and were fixed in 10% formalin for 24 h at room temperature, dehydrated, embedded in paraffin and sectioned. Tissue sections were further used for normal histopathology, immunohistochemistry and BrdU cell proliferation assay.

(1) Normal histopathology

Sections stained with hematoxylin and eosin were observed under a microscope for the micro-architectural changes (Saleh et al., 2017).

(2) Immunohistochemistry

Tissue sections were deparaffinised, rehydrated, quenched with 3% H2O2 in methanol for 1 min at room temperature, microwaved for 7 min, trypsinized for 10 min at room temperature, rinsed, and blocked with 2% goat serum. The primary antibody was diluted as: 1:10 anti-insulin and incubated for 1 h at room temperature. Secondary antibody (horseradish peroxidase (HRP) –linked) was incubated for 20 min at room temperature. The secondary antibody was developed in 3, 3′-Diaminobenzidine (DAB) as substrate (Wang et al., 2014).

Morphometry

The measurements were done with the use of Image J associated with a Leica microscope. The area percentage of insulin positive cells were evaluated (Mustafa et al., 2015).

(3) BrdU cell proliferation assay

Rats were given injections of bromodeoxyuridine (BrdU); 50 mg/kg body weight, i.p., one injection every 2 hours until 6 hours. Animals were sacrificed 24 hours after BrdU administration (Marzo et al., 2004). BrdU in tissue sections was detected using the BrdU Immunohistochemistry Kit following the manufacturer's instructions (Hino et al., 2004).

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni post hoc test. The criterion for statistical significance was at a *P*-value less than 0.05. Data was analyzed using Graph Pad, Prism software, version 5.02.

Results

In vitro

Effect of QE on MIN6 cell viability

MIN6 cells in the DC group displayed significantly reduced cell viability as compared to NC group ($P < 0.001$). MIN6 cells destroyed by STZ showed significantly ($P < 0.001$) higher viabilities on administration of QE (10µg/ml). Administration of QE (10µg/mL) to MIN6 cells without STZ did not have any significant effect on cell viability as compared to NC group. Similarly, treatment of STZ damaged cells with DMSO did not have any significant effect as compared to DC group (Figure 1).

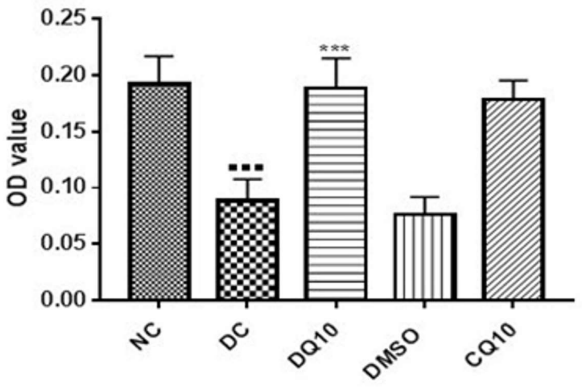

Figure 1. Effect of QE on MIN6 cell viability. Figure Shows significantly ($P < 0.001$) decreased cell viability in the DC group as compared to NC group. DQ10 significantly ($P < 0.001$) increased cell viability as compared to DC group. No significant effect was observed on cell viability in group CQ10 as compared to NC group and the DMSO group as compared to DC group. ■■■$P <$ 0.001 *vs* NC group; ***$P < 0.001$ *vs* DC group.

***In vivo* study**

Effect of QE on body weight

There was no significant difference in the initial body weights of rats. However, the final body weight of the NC group was significantly higher than that of the DC group. Quercetin administration (25, 50,100 mg/kg) significantly increased body weights as compared to DC group (Table 1).

Table 1. Body weights in control and experimental group

Groups	Body weight (g)	
	Initial	Final
NC	192.4± 6.242	298.8±4.883
DC	200.4±4.4	172.2±4.14▪▪▪▪
DQ25	208.4±5.046	211±5.431****
DQ50	205±4.324	219.8±3.625****
DQ100	193.8±6.344	214.6±5.644****

The data show no significant difference in initial body weights of all groups. Significant ($P < 0.0001$) decrease in final body weight of the DC group as compared to NC group. Significant increase ($P < 0.0001$) in body weights of DQ25, DQ50, DQ100 group as compared to DC group. ▪▪▪▪ $P < 0.0001$ *vs* NC group; **** $P < 0.0001$, *vs* DC group.

The effect of QE on fasting blood glucose

The Blood glucose level was significantly ($P < 0.0001$) increased in the DC group as compared to NC group. Administration of QE (50mg/kg) and QE (100mg/kg) significantly ($P < 0.0001$) decreased the blood glucose level as compared to DC group. QE (25mg/kg) did not have any significant effect as compared to DC group (Figure 2).

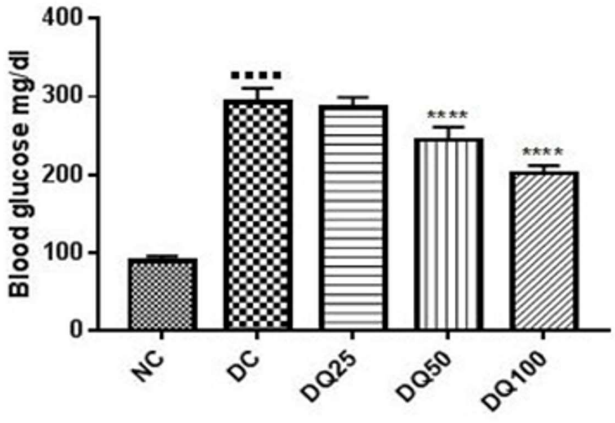

Figure 2. Effect of QE on fasting blood glucose. Figure showed Blood glucose level significantly increased ($P < 0.0001$) in the DC group as compared to NC group. Significant blood glucose reduction was observed in DQ50 ($P < 0.0001$) and DQ100 ($P < 0.0001$) group; no significant effect was observed in DQ25 group as compared to DC group. ▪▪▪▪ $P < 0.0001$ *vs* NC group; **** $P < 0.0001$ *vs* DC group.

Effect of QE on Serum insulin

Serum insulin was significantly ($P < 0.0001$) decreased in the DC group as compared to NC group. QE administration, 50mg/kg and 100mg/kg significantly elevated serum insulin level ($P < 0.001$), ($P < 0.0001$) respectively, as compared to DC group. No significant effect was noted with administration of QE (25mg/kg) as compared to DC group (Figure 3).

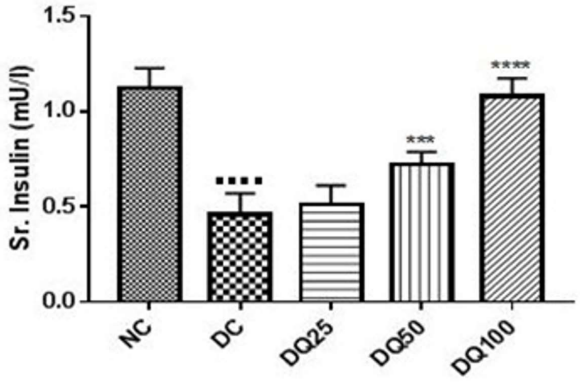

Figure 3. Effect of QE on Serum insulin. Figure Shows significantly decreased ($P < 0.0001$) serum insulin level in the DC group as compared to NC group. Significant increase in serum insulin was observed in DQ50 ($P < 0.001$) and DQ100 ($P < 0.0001$) group; no significant effect was observed in DQ25 groups as compared to DC group. ▪▪▪▪ $P < 0.0001$ *vs* NC group; **** $P < 0.0001$, *** $P < 0.001$ *vs* DC group.

Effect of QE on histological examinations

Effect on normal histopathology

Degeneration of islets, pancreatic damage and reduction in β-cells was observed in the DC group as compared to NC group. Administration of QE (50mg/kg) and QE (100mg/kg) resulted in the regeneration and restoration of islets followed by increased β-cells number and reduced pancreatic damage as compared to DC group. No notable changes were observed in pancreatic tissue sections of DQ25 group as compared to DC group (Figure 4).

Effect on immunohistochemistry

Pancreatic insulin secretion significantly ($P < 0.0001$) decreased in the DC group as compared to NC group. Administration of QE (100mg/kg) significantly ($P < 0.0001$) elevated insulin secretion as compared to DC group. QE (25mg/kg & 50mg/kg) did not have any significant effect on insulin secretion as compared to DC group (Table 2 and Figure 5).

Effect on BrdU cell proliferation assay

The increased number of BrdU positive cells was observed on QE (100mg/kg) administration as compared to the DC group (Figure 6).

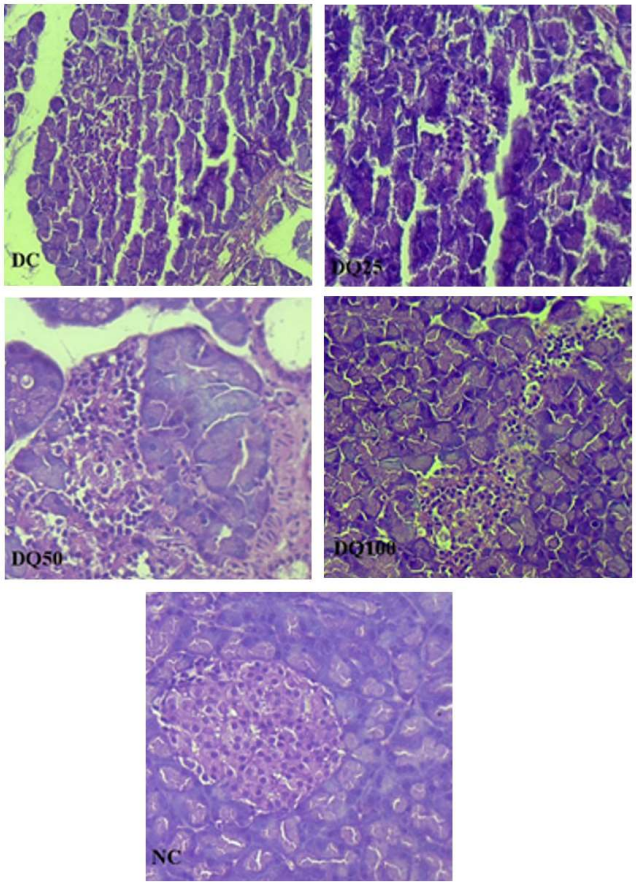

Figure 4. Effect on normal histopathology. DC group showed pancreatic damage and reduction in β-cell number as compared to NC group. Regeneration and restoration of islets, with a subsequent increased number of β-cells was observed in DQ50 and DQ100 group as compared to DC group. Significant changes were not observed in pancreatic tissue of DQ25 group as compared to DC group.

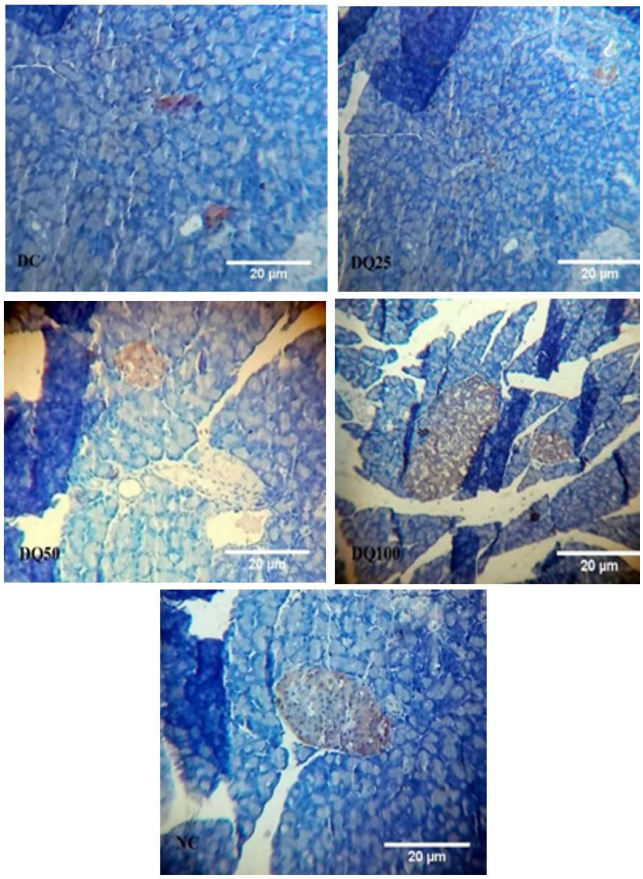

Figure 5. Effect on immunohistochemistry. Immunohistochemistry staining of insulin (brown). Insulin secretion was significantly ($P < 0.0001$) decreased in the DC group as compared to NC group. Significant ($P < 0.0001$) increase in insulin secretion was observed in DQ100 group as compared to DC group. DQ25 and DQ50 group did not have any significant effect on insulin secretion as compared to DC group.

Table 2. The area percentage of insulin positive cells

Groups	%Area
DC	0.6228±0.0675■■■■
DQ25	0.2645±0.0166
DQ50	1.562±0.2437
DQ100	8.007±0.2948****
NC	7.681±0.3756

The data show significant ($P < 0.0001$) decrease in a % Area of insulin positive cells in the DC group as compared to NC group. Significant increase ($P < 0.0001$) in % Area was observed in DQ100 group as compared to DC group. No significant difference in % Area was observed in DQ25 and DQ50 group as compared to DC group. ■■■■$P <$ 0.0001, vs NC group; **** $P < 0.0001$, vs DC group.

Discussion

Functional β-cell destruction is fundamental in type 1 and type 2 diabetes. The process of beta cell destruction is assumed to be mediated by autoimmunity (Saleh et al., 2017). Oxidative stress is elevated in diabetes mellitus due to an elevation in the production of oxygen free radicals and a deficiency in antioxidant defense mechanisms. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions (Bakhshaeshi et al., 2012), thus was selected as a drug for the present study. Research work was initiated with the i*n vitro* evaluation in which STZ damaged MIN6 cells was utilized. STZ leads to elevated generation of reactive oxygen species (ROS) leading to degeneration and necrosis of β-cells. Administration of QE (10µg/ml) successfully reversed the effect of STZ leading to increased cell viability of STZ damaged MIN6 cells. In vitro study results confirmed earlier reports stating QE as a strong antioxidant (Coskun et al., 2005). Diabetes mellitus is characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia and decreased insulin secretion in diabetes is associated with long-term

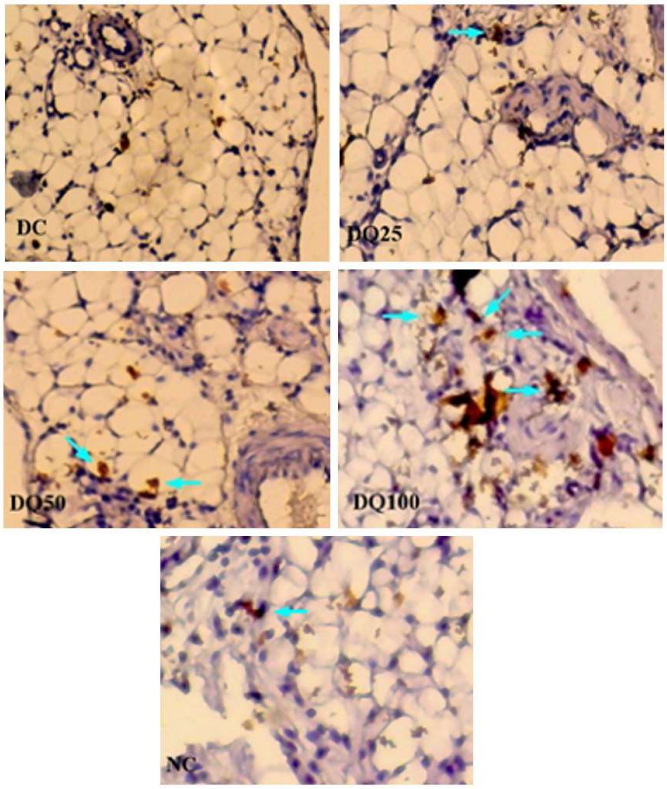

Figure 6. Effect on BrdU cell proliferation assay. Figure shows increased number of BrdU positive cells (brown) in DQ100 group as compared to DC group. Arrows indicate BrdU positive cells.

dysfunction, damage and failure of various organs in the body, particularly the nerves, kidneys, eyes, heart, pancreas itself and blood vessels (Koeslag et al., 2003). Hyperglycemia is also associated with fast depletion of pancreatic insulin stores, temporal changes in β-cell proliferation that culminates in disturbed islet cell (Mello et al., 2015). Groups DQ50 and DQ100 significantly decreased blood glucose levels and significantly elevated serum insulin level in the *in vivo* study. The Tyrosine kinase inhibitors exert antihyperglycemic effects that can reverse or prevent type I and II diabetes mellitus (Fountas et al., 2015). Our study confirmed the results of earlier reports which state that QE acts as a tyrosine kinase inhibitor to produce anti-diabetic effect (Bentz., 2009). The BrdU proliferation assay was performed to determine beta cell regenerating activity of QE. BrdU is incorporated into the newly synthesized DNA of replicating cells, substituting for thymidine during DNA replication. Antibodies specific for BrdU are then used to identify the incorporated chemical, thus indicating actively DNA replicating cells (Konishi et al., 2011). DQ100 group showed increase in the number of BrdU stained cells elevated pancreatic insulin secretion, thus confirming regeneration of beta cells. Oxidative stress leads to β-cell destruction (Aguirre et al., 2011). QE has been reported to decrease lipid peroxidation, and increase antioxidant enzymes (Coskun, 2005) resulting in a depletion of oxidative damage to cells. Quercetin thus protects cells undergoing oxidative stress and prevents Ca2+dependent cell death (Buko et al., 2016) thus supporting beta cell regeneration. The regeneration processes are induced by replication of pre-existing beta-cells, neogenesis from endogenous progenitors or *Tran's* differentiation from differentiated non-beta cells (Guz et al., 2001). Earlier reports state antidiabetic activity of Quercetin by intraperitoneal administration (Ahmed et al., 2010). The protective role of QE against STZ induced oxidative damage has been reported, wherein QE was administered before STZ intraperitoneally (Buko et al., 2016).

Thus the present study confirms antidiabetic activity of QE through beta cell regeneration on oral administration. The *in vitro* study confirmed recovery of STZ damaged MIN6 cells after QE administration, therefore proving the role of QE in β cell regeneration *in vitro* as well as *in vivo*. Quercetin activity was found to be dose dependent, significant effect was observed at the highest dose of 100mg/kg.

Declarations of interest

None

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