An immunohistochemical and biochemical evidences of pancreatic β-cell regeneration in type 2 diabetic rats after treated with Aegle marmelos leaf extract and Aegeline

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ABSTRACT

The study aims to investigate the Immunohistochemical changes in pancreatic beta cells in fructose fed, streptozocin (STZ) induced Type 2 Diabetes (T2DM) rats treated with various doses of leaf extract of Aegle marmelos (AAM) and Aegeline (AG). 42 adult male wistar albino rats were separated into 7 groups, including Vehicle Control (VC); T2DM; T2DM + AAM 250mg/kg; T2DM + AAM 500mg/kg; T2DM + AG 20mg/kg; T2DM + AG 50mg/kg and T2DM + AG 100mg/kg. Experimental T2DM was created by a single dose of 40 mg/kg STZ injection intra-peritoneally along with 10% of fructose solution given orally for 30 days. Calculated dosages of AAM and AG were given with oral gavage for 30 days. Pancreas was harvested and processed. Slides were stained using hematoxylin and eosin (H&E) stains. Insulin expressing beta-cells was analyzed using immunohistochemistry. Fructose fed, STZ induced rats showed degenerative expressions in beta-cells. In STZ treated rats, AG reduced the blood glucose concentration and serum insulin levels at the maximum functional dose compared to AAM. The immunohistochemical information suggests that the AG at 100 mg/kg dose has the capability of making the dormant cells to reproduce to restore the lost cells of islets of Langerhans.

INTRODUCTION

Diabetes mellitus (DM) has a great impact on mortality and morbidity globally (Lozano et al., 2010; Murray et al., 1990). Majority of the population live in low and middle income countries and it was calculated as 55% rise will be there in a number of diabetes worldwide by the year 2035 (International Diabetes Federation, 2012). T2DM contributes the majority of the portion in the total percentage of diabetes approximately about 90-95% (American Diabetes Association, 2013). Obesity, sedentary lifestyle and consumption of unhealthy foods are the major factors for the pandemic of T2DM, which affects expectations of life and health care cost. The T2DM is defined by the presence of chronic hyperglycemia which is a progressive and partly understood metabolic disease (American Diabetes Association, 2012). Insulin resistance with reduced insulin sensitivity and defective insulin secretion due to
decreased beta cell mass or dysfunction is the characteristic pathogenic feature of T2DM (DeFronzo, 2009). Based on this, the T2DM patients may have predominant insulin secretory defect with minimal to moderate insulin resistance or predominant insulin resistance with minimal to moderate insulin secretory defect (American Diabetes Association, 2013). Even though great progress has been achieved in the management of T2DM in recent years there still some constraints in the safety of drugs like safety, durability, glycemic variability, hypoglycemic episodes, keto acidosis, weight gain, GI disturbances etc., (Goldberg et al., 2008).

In many low and middle income countries, the affordability for the oral hypoglycemic drugs and insulin is far unreachable by many people which leads to looking for other alternatives which will be cheaper and accessible. In that context, the plant products seem to be a potential alternative to be explored for treatment of T2DM due to their abundant availability in nature, universal acceptability and relatively low side effects. Aegle marmelos (AM), which is popularly known as "Bael" in Bangla and "Wood apple" in English, is being used as one of the traditional medicine in India and Bangladesh to treat many disorders. Its leaves, fruits and roots all area used for treatment of various illness (Maity et al., 2009). More than 1001 chemical compounds have been identified, which includes marmelosin, psoralen, tannin, aurapten and luvangetin on various parts of the plants (Maity et al., 2009).

On preliminary research, it has been identified as different extracts of AM has got a glucose lowering effect along with that insulin secretary and anti-hyperlipidemic effect also noted. In most of the research alcoholic or aqueous extract of AM is being used in type 1 diabetic animals (T1DM) induced by either STZ or alloxan (Kumar et al., 2013; Gandhi et al., 2012; Narendhirakannan and Subramanian, 2010). Unfortunately, the results of such studies are not applicable in most conditions as the majority of the disease is contributed by T2DM (Saravanan et al., 2014; Sharma et al., 2011). So far, the antidiabetic effect of AM extract has analyzed to some extent and it has hypothesized that beta cell regeneration is found to be one of the possible mechanism of its hypoglycemic effect. However, it requires further research to explore other mechanisms which leads to its hypoglycemic effect. These studies require the direct manifestation of pancreatic β-cells regeneration. The present study was aimed to analyze and confirm pancreatic β-cells regeneration by AM leaf extract and its active component AG in fructose fed, STZ induced T2DM rats by biochemical and immunohistochemical method.

MATERIALS AND METHODS

Chemicals
All chemicals and reagents used in the present study were obtained from (Southern Indian scientific corporation, Chennai).

Plant material extract
The fresh leaf of AM plant was obtained at Coimbatore, Tamil Nadu in the month of June authenticated by Department of Botany, National Institute of Siddha, Chennai (Voucher no: NISMB1492014). The specimen was deposited in the National Institute of Siddha for further references. The fresh leaf of AM plant was shade dried for a week. The dried leaves were of course powdered using an electric blender; further subjected to Soxhlet extraction. An aqueous extract was prepared by dissolving 25 gm of leaf powder with 50ml of double distilled water for 48 hours. The resultant mixture was dried using rotary flash evaporator and stored in the refrigerator for phytochemical screening (Harborne, 1973).

The extracts were sequestered for the existence of various phyto compounds. Aqueous extract of AM has an appreciable amount of saponins, flavonoids, alkaloid, quinones and phenols. Carbohydrates, terpenoid, phlobatannins and anthraquinones were absent. Basically, if a reaction takes place, the changes in the solution will be positive, by means, there is the presence of a compound in the solution. The Thin layer chromatography (TLC) is used for characterizing and isolating plant bioactive compounds from the crude extract. Here the crude extract after isolation from the column chromatography is run on a TLC plate (Marston et al., 1997). The bands identified in the TLC plate represent the compound of interest, Aegeline.

Animals and experimental design
Total number of 42 adult male Wistar albino rats of weight 250–275 g was procured from TANUVAS and housed in the animal house at Tagore medical college and hospital, Chennai. This study was approved by the Institutional Animal Ethics Committee (IAEC) of Tagore Medical College and Hospital (IAEC.NO. TMC/IAEC/01/003 dated 03.12.16). The experiments were performed in accordance with the CPCSEA guidelines. The rats were housed with comfortable environment maintained at a temperature of 22-24°C, 55% humidity and also with alternate light and dark cycle periods with 12 hours interval starting at 6 AM. Animals were allowed to access the food freely. Before the commencement of the experiment, all they were acclimatized for one week.10% fructose solution was given to the rats daily. Chronic administration of the fructose solution to the rats
induces insulin resistance gradually. After 2 weeks injection STZ 40 mg/kg, a single dose was administered to the rats intraperitoneally. Because of the high instability of STZ in solution, it was prepared 10 minutes prior to injection by dissolving in 0.1 M citrate buffer (pH 4.5). Partial destruction of beta cells in islets of Langerhans happens due to Low dose of streptozocin that will lead to decreased insulin secretion after overnight fasting blood samples were taken from rat tail to measure blood glucose insulin level. Rats which showed blood glucose levels ranging between 180 to 300 mg/dl were classified as diabetic and was taken for study purpose.

**Experimental study design**

42 rats were divided randomly into seven groups (6 rats in each group). Group I -Vehicle control group (VC): VC rats received normal saline 2.0 ml/kg i.p.). Group II- Type 2 diabetic mellitus group (T2DM) - T2DM rats received a single intraperitoneal injection of STZ 40 mg along with 10% of fructose solution given orally during 30 days. Group III- T2DM + Aqueous extract of *Aegle marmelos* (T2DM + AAM -250) (250 mg/kg b.w.) group- 250 mg/kg of aqueous extract of *Aegle marmelos* leaf was treated to diabetic rats via orally during 30 days. Group IV- T2DM + Aqueous extract of *Aegle marmelos* (T2DM + AAM -500) (500 mg/kg b.w.) group- 500mg/kg of aqueous extract of *Aegle marmelos* leaf was treated to diabetic rats via orally during 30 days. Group V- T2DM + Aegeline (T2DM + AG -20) (20 mg/kg b.w.) group-20mg/kg of Aegeline compound was treated to diabetic rats via orally during 30 days. Group VI- T2DM + Aegeline (T2DM + AG -50) (50 mg/kg b.w.) group- 50mg/kg of Aegeline compound was treated to diabetic rats via orally during 30 days. Group VII- T2DM + Aegeline (T2DM + AG -100) (100 mg/kg b.w.) group- 100mg/kg of Aegeline compound was treated to diabetic rats via orally during 30 days.

### Measurements of blood glucose and serum insulin concentrations

After administration of AM and AG blood samples were drawn from each group at 2nd, 4th and 6th week after fasting for 18 hours from tail end using test strip and analyzed via blood glucose monitoring device (Accu-Check Active, Roche Diagnostics, Mannheim, Germany). At the end of experi-
mental period, all the rats were anesthetized with CO2 inhalation and blood samples were collected from the heart by cardiac puncture. Blood samples were collected slowly from the heart in order to prevent sudden collapsing since the rats were under deep anesthesia by CO2 inhalation. Blood samples was quickly collected into plain simple tubes and were allowed to clot for clotting. Centrifugation was done at 3000 RPM for 15 minutes to get a clear serum sample kept frozen (-20°C) for further analysis. With the help of automated chemical analyzer (Diastix - response 920) serum insulin concentration was measured and documented. Using serum insulin concentration and casual blood glucose the HOMA-IR scores were calculated using the formula: HOMA-IR = [Insulin (U/l) × Blood glucose (mmol/l)] / 22.5; Conversion factor: Insulin (1U/l = 7.174 pmol/l) and blood glucose (1 mmol/l = 18 mg/dl).

**Histological study**

After careful dissection of the sacrificed rats, the pancreas were collected and thoroughly washed with saline. After stretching the specimen on filter paper, it was fixed in 10% buffered formalin (pH 7.4). Then the specimen was sliced, processed and embedded into paraffin blocks which were cut into paraffin sections 4µm using a rotatory microtome. The slides were stained with Hematoxylin and Eosin (H&E) stains (Bancroft and Gamble, 2008).

**Immunohistochemical staining**

Diaminobenzidine method was used for immunohistochemistry in this study. Paraffin blocks were sectioned and attached to Poly-L-lysine coated glass slides. To enhance adhesion, the slides were dried overnight at 37°C. Sections were deparaffinized using Xylene and rehydrated sequentially in graduated ethyl alcohol. The slides were cleaned in phosphate buffer solution (PBS) two times with five minutes duration each. Then, tissues were boiled in 95-99°C citrate buffer solution for a duration of 10 min and then cooled for a duration of 20 min. Before the application of blocking serum, the tissues were washed in PBS four times for a duration of 5 minutes. After the application of the primary antibody, the tissues were incubated at room temperature overnight. Again the sections were flushed in PBS for 4 times before the application of biotinylated anti-polyvalent antibody and incubated at room temperature for a duration of 10 minutes. The secondary antibody was applied after being washed thrice in PBS and incubated at room temperature for 1 hour and again rinsed 4 times in PBS. Then the tissues were incubated in a solution of DAB (3,30 diaminobenzidines) chromogen at room temperature for a duration of 20 minutes. The incubated tissues were counter stained with haematoxylin after washing in PBS and cover slips were applied with mounting media. The immunoreactive cells were observed under a light microscope and the precision of each immuno histochemical reaction was documented as recommended by Sternberger.

**Statistical analysis**

All the results were expressed as Mean ± SEM. The statistical analysis was done using one-way ANOVA followed by Turkeys multiple comparison tests using SPSS 20.0 version, P < 0.05 was considered as significant.

**RESULTS AND DISCUSSION**

Effect of aqueous extract of Aegle marmelos leaves and its active component Aegeline on serum insulin, blood glucose and HOMA-IR (Table 1).

**Blood glucose (mg/dl)**

The blood plasma glucose level of rats in group 1 (vehicle control) was 82 ± 3.86, group 2 (T2DM) was 327.6 ± 6.85, group 3 (T2DM+ AAM 250mg) was 313.66±5.80, group 4 (T2DM+ AAM 500mg) was 292.66 ± 6.08, group 5 (T2DM+ AG 20mg) was 300 ± 3.68, group 6 (T2DM + AG 50mg) was 203.5 ± 7.20, group 7 (T2DM + AG 100mg) was 161.16 ± 4.89. T2DM group rats blood glucose level was significantly (p<0.001) increased when compared to the vehicle control group. Treatment with AAM 250/kg mg to T2DM rats shows mild changes in blood glucose level compared to T2DM group. Treatment with AAM 500mg/kg, AG 20mg/kg, AG 50mg/kg and AG 100mg/kg to T2DM induced rats exhibited significant (p<0.001) decrease in blood glucose level when compared with T2DM group. One way ANOVA showed a significant difference between the groups (F=423.17).

**Serum insulin (pmol/L)**

The blood plasma glucose level of rats in group 1 (vehicle control) was 121.7 ± 11.74, group 2...
Figure 1: Photo micro graphs of sections of the pancreas stained by H&E.

(T2DM) was 173.8 ± 4.10, group 3 (T2DM + AAM 250 mg) was 176.61 ± 2.80, group 4 (T2DM + AAM 500 mg) was 165.36 ± 2.98, group 5 (T2DM + AG 20 mg) was 169.8 ± 3.60, group 6 (T2DM + AG 50 mg) was 145.15 ± 4.60, group 7 (T2DM + AG 100 mg) was 130.33 ± 4.63. T2DM group rats serum insulin level was significantly (p< 0.001) increased when compared to the vehicle control group. No significant changes were observed in T2DM rats treated with AAM 250 mg/kg, AAM 500 mg/kg, AG 20 mg/kg to T2DM rats in serum fasting insulin level compared to T2DM group. Treatment with, AG 50 mg/kg and AG 100 mg/kg to T2DM induced rats exhibited significant (p< 0.001) lower in insulin level when compared with T2DM group. One way ANOVA showed a significant difference between the groups (F=11.7702).

HOMO-IR

The HOMO-IR values of rats in group 1 (vehicle control) was 2.165 ± 0.19, group 2 (T2DM) was 6.05 ± 0.28, group 3 (T2DM + AAM 250 mg) was 5.6 ± 0.17, group 4 (T2DM + AAM 500 mg) was 4.68 ± 0.11, group 5 (T2DM + AG 20 mg) was 4.97 ± 0.15, group 6 (T2DM + AG 50 mg) was 3.18 ± 0.10, group 7 (T2DM + AG 100 mg) was 2.71 ± 0.09. T2DM group rats HOMA IR level was significantly (p< 0.001) increased when compared to the vehicle control group. Treatment with AAM 250 mg/kg to T2DM rats shows no significant changes in HOMA IR level compared to T2DM group. Treatment with AAM 500 mg/kg, AG 20 mg/kg, AG 50 mg/kg and AG 100 mg/kg to T2DM induced rats exhibited significant (p< 0.001) decrease in HOMA IR level when compared with T2DM group. One way ANOVA showed a significant difference between the groups (F=81.8756).

Pancreas histology

The pancreatic histopathology of rats was shown in Figure 1. Normal appearance of islets of Langerhans is seen in the pancreas of vehicle control rats under a light microscope. The acinar cells are closely packed and arranged into small lobules in the exocrine part of the pancreas. Intralobular and interlobular septa separate the pancreatic lobules and the islet cells are dispersed between the acinar cells. Surrounding the acinar cells, the islet cells are seen lightly stained and the acinar cells are characterized by pyramidal shape with basal nuclei and acidophilic cytoplasm in the apical part. Normal islet cells have numerous beta cells with a regular round outline and dis-
tinct nuclei. Surrounding the islet, the connective tissue is well distinguished, giving the islet its typical spherical shape Figure 1A. In contrast, the pancreas of rats of the T2DM group showed histopathological changes in both exocrine part and endocrine part. Almost many acinar cells showed the presence of small vacuoles and also inflamed. The lining of the interlobular duct was noticed to have flattened epithelium. In STZ treated rats, the islet beta cells were completely lost Figure 1. In the group treated with AAM 250 mg/kg (group III), the pancreatic appearance showed similar features that of the T2DM group. Some islets found to be completely fibrosed Figure 1C.

In group IV, which received 500mg/kg of AAM, the islet cells showed degranulated cytoplasm in most of the cells and also dark nucleated cells in between Figure 1D. Rats treated with AG 20 mg/kg groups showed some improvement in the size of pancreatic islets and also increase in a number of beta cells. Also showed the presence of many vacuolated cells in between the interstitial tissues and also some restored islets covered by a connective sheet Figure 1E. Rats treated with AG 50 mg/kg groups showed the presence of cellular regeneration among the islet of Langerhans. The acinar cells showed lesser atrophic changes and the delineation between the exocrine and endocrine pancreas became more definite Figure 1F. Rats treated with AG 100 mg/kg groups showed marked improvement with the restoration of the normal size of islets of Langerhans and also islet cell showing an increase in the number. Most of the cells restored their shape with abundant eosinophilic cytoplasm and central small nuclei, but some of them remain with an elongated shape. The connective tissue covering the islet also regain its normal texture relatively Figure 1G.

Figure 2: Photomicrographs of insulin immuno histochemical staining of pancreatic islets (Dab 40x and 10x).
Immunohistochemical Evaluation

In the VC group, the beta cells found to be the major cell population of the islets and were concentrated mainly in the central zone. It was seen that the presence of dark brown granule in the cytoplasm is considered to be positive insulin expression Figure 2A. The T2DM group showed an increased reduction in a number of beta-cells Figure 2B. Beta-cells from group III rat (T2DM + AAM -250) appeared to be similar to that of the T2DM group Figure 2C. A slight increase in the number of immuno stained beta-cells in group IV rat (T2DM + AAM -500) was noted Figure 2D. Also, an increase in the number of reactive beta-cells was noted in group Vrat (T2DM + AG -20)Figure 2E as compared to that of the T2DM group. An increase in both the number and percentage area of reactive beta-cells in group VI (T2DM + AG -50) Figure 2F was observed, as compared to that of the T2DM group. Some islets of the group VII (T2DM + AG -100) rats showed increased beta-cell mass, while others showed percentage areas of beta-cells comparable to T2DM group Figure 2G.

DISCUSSION

T2DM is defined as a progressive disorder that pose a great threat to the human population (Steppan et al., 2001). In our study, the T2DM rats were created by continuous administration of 10% fructose solution along with a single dose of STZ (40 mg/kg) administration which produces decreased beta cell function by separating the DNA strand along with decreased insulin resistance. Many researchers have used similar T2DM animal model for many drug discoveries (Veerapur et al., 2012). Pancreatic islet showed the shrunken size and degranulated beta cells on HPE examinations in the STZ treated rats which lead to hyperglycemia because of inadequate beta cell mass. The rate of death of beta cells in STZ treated rats is higher with a subsequent decrease in the insulin secretion (Bonner-Weir et al., 2000). Cytoarchitectural changes were observed by destructed beta cells and vacuolated acini in the pancreas after STZ injection in our study and similar findings were observed by Hamden et al. (2009).

Also, STZ injection produced more damage in the beta cells of pancreatic tissues of diabetic rats like decreased islet cell numbers, including cell damage and cell death. Thickening of the blood vessels happened, causing ischemia of the tissues causing regressive changes and necrosis. These changes are mainly due to increased oxidative stress that happens in the progression of diabetes. Some of the mechanisms through which diabetic complications occur because of oxidative stress are glucose oxidation, non-enzymatic glycation of proteins and followed by oxidative degradation of glycated proteins (Maritim et al., 2003). Oral administration of AAM 250 mg/kg, AAM 500 mg/kg, AG 20 mg/kg, AG 50 mg/kg and AG 100 mg/kg to T2DM rats significantly prevented the increase in fasting blood glucose levels, which shows the beneficial effect of AAM and AG in controlling hyperglycemia and its diabetic complications. Rats treated with AG 20 mg/kg, AG50 mg/kg and AG 100 mg/kg showed normalization of serum insulin levels which may be due to effect of the compound on regenerated beta cells to secrete insulin or due to release of bound insulin from beta cells by suppressing ATP sensitive K+ channels like glibenclamide (Sunil et al., 2012).

In the present study, immunohistochemical analysis showed that the pancreatic islets have impaired and degranulated beta cells with decreased insulin positivity staining. But rats treated with AAM 500 mg/kg retained the beta cell mass. The protective role of AG in reversing the beta cell damage and inducing the amelioration in the functioning of beta cells is substantiated by an increase in insulin immuno reactive expression groups treated with AG 50 mg/kg and AG 100 mg/kg. These are consequence of protection of beta cells by AG preventing it from oxidative stress damage caused by STZ and fructose administration. It is similar to that of Coumarins, a hypoglycemic agent with insulin sensitization effect (Qin et al., 2010). In the present study rats treated with AAM 250 mg/kg showed completely fibrosed islets and AG 20 mg/kg, AG 50 mg/kg and AG 100mg/kg treated diabetic group rats showed reversal changes like increased in the diameter of islets and normalization of cells in islets. AG has the capacity to stop the oxidative stress and thereby restoring the feature of the pancreas in the treated group. In accordance with the previous study, the alterations occurred in the structure of pancreatic tissue of diabetic rats induced by STZ were corrected to near normal by AM leaf extract (Balamurugan et al., 2014). In our study, the rats treated with AG showed better recovery than AAM. AG has the capacity to restore the islet of pancreas better than AAM. This may be because of inactivating action of the free radical activity in DM induced animal, which played a major role in fast recovery in diabetic induced animal (Balamurugan et al., 2014).

In STZ diabetic animals there were an increased in lipid peroxides. This type of lipid peroxides increases the thiobarbituric acid reactive substances level, which damages the pancreatic architectures. Administration of extract decreases the TBARS concentration that will help to restore the pancreatic cell. Hydroperoxides molecules which...
produce from free radical are high toxicity potential which will destruct the enzymes and cell membranes. This type of destruction is mainly by the decrease in the antioxidant effect. This decreased in antioxidant effect will result in a diminished level of both enzymatic and enzymatic antioxidants. The biochemical parameters and the histological observations of pancreatic in our study parallels each other. In the pancreatic islet, the beta cells were degraded and reduced in quantity in diabetic rats. Even though the low dose of AAM showed no recovery from the damages produced by STZ on pancreatic islets, the high doses of AG 100 mg/kg revealed a marked reduction in the morphological alteration of pancreatic islets in diabetic rats. This can be explained by the presence of Theophylline, a metabolic product of AM, which is known as a smooth muscle relaxant and also has a vasodilator effect on pancreatic blood vessels (Rahman and Parvin, 2014).

The reduction in fasting blood glucose levels in AM treated group compared to that of diabetic rats could be due to insulin action secreted by regenerated beta cells. This hypoglycemic action of AM is comparable with that of oral sulphonylurea, which is a synthetic drug that produces hypoglycemia by stimulating the secretion of insulin from beta cells of the pancreas (Pan et al., 2009). The results also showed that AM decreases the hyperinsulinemia levels in diabetic rats showing a reduction in the insulin resistance. Therefore the hypoglycemic effect produced by AM is done by increasing the insulin secretion because of the regenerative effect on beta cells and also by reducing the insulin resistance.

CONCLUSIONS

In conclusion, the immunohistochemical study of the fructose fed STZ induced T2DM rats showed decreased insulin immunoreactive expression and treatment of those groups of rats with a high dose of AG may restore the normalization of biochemical, histological and immunohistochemical changes of beta cells in the pancreas. Also, the high dose of AG 100 mg/kg has the ability to induce the quiescent cells to regenerate and replace the lost beta cells of islets of Langerhans.

Conflict of Interest

The authors whose names are listed, certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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