Studies on Pancreatic Gene Expression in Diabetic Rats Treated with *Moringa oleifera* Leaf

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**Authors’ contributions**

This work was carried out in collaboration among all authors. Authors AN, EIO, HUN, DCN and INN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors CCNV, COA, OMTBO and AMI managed the analyses of the study. Authors CJO, GUO and MNA managed the literature searches. All authors read and approved the final manuscript.

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**ABSTRACT**

*Moringa oleifera* leaf have been used for treatment of diabetes, in this work we studied pancreatic gene expression. The gene expression was done by PCR machine. The plant was harvested from garden within Madonna University and was identified in the Department of Pharmacognosy of the

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University by Dr P. Osuagwu. Each of the extracts was analyzed for bioactive components using Gas Chromatography–Mass Spectrometry (GC-MS). Male wistar albino rats (n=40) six weeks old weighing 150-250g were purchased from the animal farm of Madonna University Elele. From this work we observed that, there was significant increase in the expression of ADIPOQ gene in the diabetic rats treated with 300mg/kg of M. oleifera leaf (1.52±0.13), when compared with that of the non-diabetic rats (1.00±0.02). From this study, it could be inferred that M. oleifera leaf powder contains polyphenols that inhibit carbohydrate metabolism, reduced blood glucose level, enhance expression ADIPOQ genes.

Keywords: Pancreatic gene expression; diabetic rats; Moringa oleifera leaf.

1. INTRODUCTION

Diabetes mellitus is a disease characterized by hyperglycemia resulting from impairment of insulin secretion, release, transportation, stimulation and insulin action. Uncontrolled increase in blood glucose levels may lead to macro/microvascular complications, such as cardiovascular diseases, kidney diseases, hyperlipidemia, nephropathy, neuropathy etc. For prevention of these complications, blood sugar level control using antidiabetic medications and diet are very necessary. Diet control may be achieved using orthodox medication or herbal medication. Many plants are consumed for therapeutic purposes owing to their nutritional and bioactive constituents. Moringa oleifera is one of the plants used for control of diabetes; its leaves are rich in bioactive compounds such as polyphenols, vitamins, and many other important minerals (Misra et al, 2011).

Moringa oleifera belongs to the family Moringaceae, the order Brassicales, and the genus Moringa, there are 13 species of the plant ranging in height from 5 to 10 m. It has an open crown of drooping, feathery foliage, flowers with distinctive green patches at the tips of the petals and sepals, tripinnate leaves and trunk. It’s flowers, pods, and leaves have medicinal benefits owing to various phytochemical constituents. The flower is used to treat inflammation for its stimulant content, the spots and seeds have liver-protective and antihypertensive properties, while the leaves are used to treat microbial infections and to control blood glucose levels. Moringa oleifera contains soluble fibers that enhance reduction of glucose levels, proliferation of lymphocytes and induced nitric oxide from macrophages. The leaves contains polyphenols and has been found to be useful in diabetes conditions because of their possible capacity to decrease blood glucose concentrations [1,2].

Diabetes mellitus is classified into four broad categories: gestational, type 1, type 2 and "other specific types". The "other specific types" are a collection of a few dozen individual causes. Diabetes is a more variable disease than once thought and people may have combinations of forms. The term "diabetes", without qualification, refers to diabetes mellitus. Type 1 diabetes is characterized by loss of pancreatic beta cells, leading to insulin deficiency. Majority of type 1 diabetes is of the immune-mediated nature, the immune system cell (killer T-cells) attack the pancreatic tissue leads to the loss of beta cells of the pancreas, resulting in reduced insulin synthesis and secretion. Cells sensitivity and responsiveness to insulin are usually normal, especially in the early stages. This type of diabetes also refers to as "juvenile diabetes" owing to its frequent onset in early stages of life [1,2].

Blood glucose homeostasis is controlled by the pancreas; this is an endocrine gland responsible for producing several hormones, such as insulin, glucagon, somatostatin and pancreatic polypeptide. Its exocrine function involves the secretion of digestive enzymes that aid the digestion and absorption of nutrients in the small intestine. Pancreatic function is controlled by several genes which includes; GLP-1 RECEPTOR (glucagon-like peptide-1 receptor) gene, RECTIFYING (Kv2.1/2.2) gene, SNAP23/25 gene, ADIPOQ gene, L-(CaV1.3) gene.

GLP-1 RECEPTOR (glucagon-like peptide-1 receptor) gene produces instruction for producing GLP-1R, it is located at position 6 in human chromosome. It is highly expressed in pancreatic beta cells, activated GLP-1R stimulates the adenylyl cyclase pathway which result in increase insulin synthesis and the release of insulin. RECTIFYING (Kv2.1/2.2) gene are highly expressed in pancreatic islet, it is involved in the regulation of glucose-induced
insulin secretion in the beta cells. L-(CaV1.3) mediates the depolarization to induce calcium influx in insulin secreting cells, they are highly expressed at the human beta cells, they are essential in generation of electrical activities in the cells. , SNAP25 gene are present in the beta cell of the pancreas, SNAP23/25 gene is involved in the release of insulin secretion while, SNAP23 promotes vesicle fusion in pancreatic cells. ADIPOQ gene code for adiponectin synthesis, adiponectin is secreted by adipocytes and is highly expressed in human plasma, it regulates the peripheral glucose and fatty acid, it increases insulin sensitivity of the target organs; the liver and the muscle.

Intestinal availability of glucose is controlled by the carbohydrate digestive enzymes such as alpha glucosidase and alpha amylase. Competitive or reversible Inhibition of these intestinal enzymes by some ligands slows the digestion of carbohydrate and delays glucose absorption. This results in a smaller and slower rise in blood glucose following carbohydrate meal. Intestinal cells also produce incretins such as glucagon-like peptide-1, which functions to regulate blood glucose level by enhancing insulin production and regulation of appetite. This protein is inhibited or degraded by dipeptidyl peptidase-4 (DPP-4), inhibition of DPP-4 reduces glucagon and blood glucose by increasing the incretin levels (GLP-1), which inhibit glucagon and increase insulin secretion, decrease gastric emptying, and decreases blood glucose levels [3].

The study was done to determine of pancreatic expression of ADIPOQ gene, Glucagon-like peptide-1 gene (GLP-1 RECEPTOR), RECTIFYING (Kv2.1/2.2) gene, Calcium voltage gated channel L-(CaV1.3), SNAP23/25 gene in the rats after treatment with pulverized Moringa oleifera leaf.

2. METHODS

2.1 Plant Materials and Preparation

The plant was harvested from garden within Madonna University and was identified in the department of pharmacognosy of the University by Dr P. Osuagwu. The leaves were air dried at room temperature for two weeks, after which it was pulverized using electronic blender, the pulverized sample was subjected for extraction using four different solvents namely; ethanol, ethyl acetate, hexane and water. Each of the extracts was analyzed for bioactive components using Gas Chromatography – Mass Spectrometry (GC-MS).

2.2 Animals Handling

Male wistar albino rats (n=40) six weeks old weighing 150-250g were purchased from the animal farm of Madonna University Elele. Each of the animals was housed in animal cage with wire mesh and saw dust lining, and they were kept in a room inside the animal house, with 12 hours light/dark circle. The animals were allowed to acclimatize for 2 weeks, and were given food and water.

2.3 Experimental Design

After two weeks, they were numbered and separated into four groups of 10 rats each, group one were fed with animal feed throughout the experimental period, while other groups were fed with high fat diet (HFD) for seven weeks to increase the body mass index. At the end of the 9th week, 0.5ml of of streptozotocin 37mg/kg body weight in citrate buffer was administered intraperitoneally to the rats in groups 2, 3 and 4. The rats in groups 3 and 4 in addition to streptozotocin were fed with pulverized Moringa oleifera leaf daily with the aid of rats cannular, according to the experimental design below. Fasting blood sugar was measured weekly by cutting the tip of the animals tail, using Easy Touch HealthPro glucose monitoring system [4].

Group 1 (Negative control): The animals in this group were fed with only animal mesh and water throughout the experiment.

Group 2 (Positive control): The animals in this group were given 0.5ml of 37mg/kg of Streptozotocin intraperitoneally in addition to feed and water.

Group 3: The animals in this group were given 0.5ml of 37mg/kg of streptozotocin and 150mg/kg of Moringa oleifera leave powder daily, in addition to food and water throughout the experiment period.

Group 4: The animals in this group were given 0.5ml of 37mg/kg of streptozotocin and 300mg/kg of Moringa oleifera leave powder daily, in addition to food and water throughout the experiment period.
2.4 Determination of Lethal Dose

This involves two steps; in the first step, nine animals were used grouped into three animals, each group were given different doses of the *Moringa oleifera* leaf powder (50, 100, 150mg/kg). The animals were monitored for 24 hours. Second step three groups of one animal each were given different higher doses of *Moringa oleifera* leaf powder (200, 300, and 400mg/kg). The animals were monitored for 24 hours.

LD50 was determined using the formular;

$$LD50= \sqrt{D_o \times D_{100}}$$

Where $D_o$ = the highest dose that gave no mortality

$D_{100}$ = the lowest dose that produced mortality

2.5 Induction of Diabetes Mellitus in Rats

Diabetes mellitus was induced by intraperitoneally injecting the rats with STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 37 mg/kg body weight (b.w.) after two weeks of adaptation and seven weeks of feeding with high fat diet. STZ was freshly prepared as solution in 10 mM sodium citrate buffer (pH 4.5) and injected to after overnight fasting. Fasting blood glucose was measured before injection. On the third day after the STZ injection, the blood was sample was collected from the tail of STZ-injected animals, and glucose levels were measured using glucometer (Easy Touch HealthPro glucose monitoring system).

2.6 Sample Collection

At the end of the experimental period, the animals were euthanized by exposure to chloroform, blood sample was collected via cardiac puncture. Blood was collected into test tubes labeled accordingly. Serum samples were separated and used for determination of different biochemical parameters. Liver and kidney were surgically removed. Liver and kidney were washed with ice cold (4°C) phosphate buffer saline (immediately after removal) to remove blood, tissue homogenate was prepared by homogenization of 1g of liver/ kidney using BeadBug 6 position tissue homogenizer, the remaining part of the tissue was preserved using formalin for histological studies.

2.7 Total RNA Isolation

Total RNA was isolated from freshly excised pancreatic tissues following a method described by Omotuyi et al. [5]. Tissues were homogenized in cold (4 °C) TRI reagent (Zymo Research, USA, Cat:R2050-1-50, Lot: ZRC186885). Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) in 30 ml of nuclease-free water (Ingaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML)). The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, pH 6.4).

2.8 cDNA Conversion

Prior to cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 × $A_{260}$) and quality ($\geq$ 1.8) was assessed using the ratio of $A_{260}/A_{280}$ (A=absorbance) read using spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNase I treatment (NEB, Cat: M0303S) as specified by the manufacturer. 2 µl solution containing 100 ng DNA-free RNA was converted to cDNA using M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) in 20 µl final volume (2 µl, N° random primer mix; 2 µl, 10X M-MuLV buffer; 1 µl, M-MuLV RT (200 U/µl); 2 µl, 10 mM dNTP; 0.2 µl, RNase Inhibitor (40 U/µl) and 10.8 µl nuclease-free water). The reaction proceeded at room temperature Q/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C/20 min.

2.9 PCR Amplification and Agarose Gel Electrophoresis

PCR amplification for the determination of genes whose primers (SnapGene software) are listed below (table 1.0) was done using the following protocol: PCR amplification was performed in a total of 25 µl volume reaction mixture containing 2 µl cDNA (40 ng), 2 µl primer (100 pmol) 12.5 µl Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial
Table 1. List of primers for ADIPOQ, SNAP23, SNAP25, AND GLP-1R GENE

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Accession number</th>
<th>Length forward</th>
<th>Length reverse</th>
<th>Forward primer sequence(5'-3')</th>
<th>Reverse primer sequence(5'-3')</th>
<th>Optimum TM</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novegicus potassium voltage gated channel subunit alpha D (mRNA) Subfamily B Number1</td>
<td>NM 013186.1</td>
<td>17</td>
<td>20</td>
<td>GGTGGCCGCCAAGATCC</td>
<td>CGAACTCGTCTAGGC TCTGC</td>
<td>60°C</td>
<td>107bp</td>
</tr>
<tr>
<td>Calcium voltage gated channel subunit alpha D (mRNA)</td>
<td>NM 1017298.1</td>
<td>20</td>
<td>20</td>
<td>ATCCAAAGCTCAGATCGC</td>
<td>GTGGGCTGAGAACC TAGACG</td>
<td>59°C</td>
<td>100bp</td>
</tr>
<tr>
<td>Snaptosome associated protein25 (mRNA)</td>
<td>NM 012728.1</td>
<td>18</td>
<td>20</td>
<td>GGATGAGCAAGGCGAGAC ACG</td>
<td>ACGGGACAGCATTTG</td>
<td>60°C</td>
<td>111bp</td>
</tr>
<tr>
<td>Glucagon-like peptide receptor (GLP1R) mRNA</td>
<td>NM 012728.1</td>
<td>20</td>
<td>20</td>
<td>ACAGGTCTCTTCTGCAA CGG</td>
<td>ATGCCCTTGAGACAC ACTAC</td>
<td>61°C</td>
<td>126bp</td>
</tr>
<tr>
<td>Adiponectin, CIQ and collagen domain containing (Adipoq) mRNA</td>
<td>NM 144744.3</td>
<td>20</td>
<td>20</td>
<td>CCACCCAAGGAAACTTG TGC</td>
<td>GACCAAGGACACCTG CGTCT</td>
<td>61°C</td>
<td>136bp</td>
</tr>
<tr>
<td>Snaptosome associated protein 23 (Snap23) mRNA</td>
<td>NM 022689.2</td>
<td>20</td>
<td>20</td>
<td>GGGCTCCTGCTGAGTCTTTT</td>
<td>ACGGCTGAGCAAGAA AGGCTGAGCAAGAA</td>
<td>61°C</td>
<td>116bp</td>
</tr>
<tr>
<td>Actin beta (Actb) mRNA</td>
<td>NM 031144.3</td>
<td>20</td>
<td>20</td>
<td>CTGGCTCCTAGCACCATT GAA</td>
<td>CCTAC CGCAGCTCAGTAACA GTCCG</td>
<td>61°C</td>
<td>192bp</td>
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</tbody>
</table>
Table 2. The MEAN±STD of the pancreatic ADIPOQ, SNAP23, SNAP25, Kv2.1 and GLP-1R gene expression of all the rats in the study

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>ADIPOQ</th>
<th>SNAP25</th>
<th>SNAP23</th>
<th>Kv2.1</th>
<th>GLP-1R</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP1</td>
<td>1.00±0.02</td>
<td>1.00±0.02</td>
<td>1.00±0.04</td>
<td>1.00±0.04</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>GROUP2</td>
<td>1.12±0.11</td>
<td>0.85±0.09</td>
<td>1.03±0.10</td>
<td>1.09±0.11</td>
<td>0.62±0.09</td>
</tr>
<tr>
<td>GROUP3</td>
<td>1.16±0.12</td>
<td>1.02±0.11</td>
<td>1.03±0.11</td>
<td>1.19±0.11</td>
<td>0.66±0.08</td>
</tr>
<tr>
<td>GROUP4</td>
<td>1.52±0.13</td>
<td>0.81±0.09</td>
<td>1.15±0.13</td>
<td>1.15±0.12</td>
<td>0.76±0.09</td>
</tr>
</tbody>
</table>

P VALUE 0.04 0.29 0.10 0.06 0.30 (0.05)

Fig. 1. ADIPOQ gene expression

Fig. 2. SNAP-25 gene expression
Fig. 2. SNAP23/25 gene expression

Fig. 3. L-(CaV1.3) gene expression

Fig. 4. Kv2.1/2.3 gene expression
denaturation at 95 °C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing (see TM values for each primer pair on table 1.0) for 30 seconds and extension at 72 °C for 60 seconds) and ending with final extension at 72 °C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811) in Tris (RGT reagent, china, Lot: 20170605)-Borate (JHD chemicals, China, Lot 20141117)-EDTA buffer (pH 8.4).

2.10 Amplicon Image Processing and Semi-Quantification

In-gel amplicon bands images captured on camera were processed on keynote platform. Gel density quantification was done using Image-J software [6]. Each point represent relative expression (test gene band intensity/ internal control band intensity*100) plotted using Numbers software (Mac OSX version).

The table above shows the number of primers used for each of the gene both forward and reverse primers.

3. RESULTS

From this table it could be observed that there is no significant difference in the pancreatic expression of the SNAP25, SNAP23, Kv2.1 and GLP-1 genes in all the rats treated in the experiment. But, there is a significant increase in the pancreatic ADIPOQ gene expression of the diabetic rats in group 4 (1.52±0.13) treated with 300mg/kg of Moringa oleifera leaf powder, when compared to the non-diabetic rats in groups 1, and untreated diabetic rats in group 2 (1.00±0.02 and 1.12±0.11) respectively.

4. DISCUSSION

Furthermore, from the results obtained from estimation of the pancreatic gene expression, there is a significant increase in the expression of ADIPOQ gene in the diabetic rats treated with 300mg/kg of Moringa oleifera leaf (1.52±0.13), when compared with that of the non-diabetic rats (1.00±0.02). But there is no significant difference in the pancreatic expression of the ADIPOQ gene among the diabetic rats treated with Moringa oleifera leaf and the untreated diabetic rats. An ADIPOQ gene code for adiponectin, adiponectin is secreted by the adipocyte. The treated and untreated diabetic rats groups were fed with high fat diet at the beginning of the experiment, and they all have increased body mass index, this may be the reason for the expression of ADIPOQ gene seen among these groups. This is evidenced by the report from the findings of Wade et al. [7] who reported no significant difference in the pancreatic gene expression of ADIPOQ2 among lean and obese mice. We also found a significant increase in the expression of SNAP-23 gene among the diabetic rats treated with 300mg/kg of Moringa oleifera leaf (1.51±0.13), when compared with that of the non-diabetic rats and untreated diabetic rats (1.00±0.04 and 1.03±0.10) respectively. It had been found that SNAP-23 regulates a wide variety of regulated and constitutive-exocytosis in various cells such as the adipocytes, and pancreatic acinar cells (Foster et al., and Huang
et al., 2001). The increased expression of SNAP-23 could be attributed to the activity of the gene in stimulating the release of adiponectins and leptin from the adipocytes. This is in agreement with the investigated evidence by Samar et al. [8], who reported that *Moringa oleifera* reduced total cholesterol, triglyceride, low density cholesterol, high density cholesterol and very low density cholesterol in rats and that it is more effective in prevention of hyperlipidemia. We also found no significant difference in the expression of SNAP25, Kv1.2, and GLP-1R genes among all the rats. It could be seen that the plant has no effect on the expression of these genes that regulate insulin synthesis and stimulation.

5. CONCLUSION

The study revealed increase in the expression of ADIPOQ gene in the diabetic rats treated with 300mg/kg of *M. oleifera* leaf (1.52±0.13), when compared with that of the non-diabetic rats. It could be inferred that *M. oleifera* leaf powder contains polyphenols that inhibit carbohydrate metabolism, reduced blood glucose level, enhance expression ADIPOQ genes.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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