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Decreased muscarinic M1 receptor gene expression in the cerebral cortex of streptozotocin-induced diabetic rats and *Aegle marmelose* leaf extract's therapeutic function

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Abstract

Aim: In the present study we have investigated the changes in the total muscarinic and muscarinic M1 receptor ([^{3}H]QNB) binding and gene expression in the cerebral cortex of streptozotocin (STZ) induced diabetic, insulin and aqueous extract of *Aegle marmelose* leaf treated diabetic rats.

Materials and Methods: Diabetes was induced in rats by intrafemoral injection of streptozotocin. *Aegle marmelose* leaves was given orally to one group of rats at a dosage of 1 g/kg body weight per day for fourteen days. Blood glucose and plasma insulin level were measured. Muscarinic and Muscarinic M1 receptor binding studies were done in the cerebral cortex of experimental rats. Muscarinic M1 receptor gene expression was studied using real-time PCR.

Results: Scatchard analysis for total muscarinic receptors in cerebral cortex showed that the B_{max} was decreased significantly ($p < 0.001$) in diabetic rats with a significant decrease ($p < 0.01$) in the K_d when compared to control group. Binding analysis of Muscarinic M1 receptors showed that B_{max} was decreased significantly ($p < 0.001$) in diabetic group when compared to control group. The K_d also decreased significantly ($p < 0.01$) when compared to control group. The binding parameters were reversed to near control by the treatment of diabetic rats with *Aegle marmelose*. Real-Time PCR analysis also showed a similar change in the mRNA levels of muscarinic M1 receptors.

Conclusion: The results showed that there is decrease in total muscarinic and muscarinic M1 receptors during diabetes which is up regulated by insulin and *Aegle marmelose* leaf extract treatment. This has clinical significance in therapeutic management of diabetes.

Keywords: Muscarinic receptors; Cerebral cortex; Streptozotocin; *Aegle marmelose*; Diabetes

1. Introduction

Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Amos et al., 1997; Zimmet, 1999; Zimmet et al., 2001). The number of diabetic patients is expected to reach 300 million by the year 2025. (Adeghate et al., 2006). The brain neurotransmitters receptor activity and hormonal pathways control many physiological functions in the body. Earlier studies from our laboratory have established the central neurotransmitter receptor subtypes functional regulation during diabetes, pancreatic regeneration and cell proliferation (Paulose et al., 1988; Sudha and Paulose, 1988; Abraham and Paulose, 1999; Paulose et al., 1999; Biju et al., 2001; Renuka et al., 2004; Mohanan et al., 2005a, b; Kaimal et al., 2007). The central nervous system through parasympathetic and sympathetic pathways regulates insulin secretion from pancreatic islets and maintains glucose homeostasis (Ahrén, 2000). Acetylcholine (ACh), the major parasympathetic neurotransmitter stimulates the glucose-induced insulin secretion (Gilon and Henquin, 2001). It has been demonstrated that the cholinergically induced insulinotropic action is mediated by the muscarinic

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receptors (Henquin and Nenquin, 1988). It has been proposed that neuronal afferent signals delivered to the pancreatic β cell through the vagus are responsible for the cephalic phase of insulin secretion. These effects are mediated by ACh, which is released from nerve terminals and acts upon muscarinic cholinergic receptors in the β cell plasma membrane (Sharp et al., 1974; Berthoud et al., 1980; Mathias et al., 1985; Ahrén, 2000).

Plants still remain a major source for drug discovery in spite of the great development of synthetic molecules. Consequently, the uses of traditional plant extract in the treatment of various diseases have been flourished (Fabricant and Farnsworth, 2001). The plant *Aegle marmelose* (L.) Corr. belongs to the family Rutaceae and is known as 'Koovalam' in Malayalam and bael in Hindi is indigenous to India. Leaves, fruits, stem and roots of *Aegle marmelose* have been used in ethno medicine for several medicinal properties: astringent, antidiarrheal, antidysenteric, demulcent, antipyretic, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom (Kirtikar and Basu, 1935; Nandkarni, 1976). *Aegle marmelose* is also known as herbal medicine for the treatment of diabetes mellitus (Alam et al., 1990; Prakash, 1992). Ponnachan et al. (1993) have observed that the crude aqueous leaf extract (1 g/kg) exhibit hypoglycemic effect in alloxan-induced diabetic rats. Aqueous leaf extract reversed the increase in K_m values of liver malate dehydrogenase enzyme (Seema et al., 1996) and improved histopathological alterations in the pancreatic and kidney tissues of streptozotocin (STZ)-induced diabetic rats (Das et al., 1996). The aqueous extracts of fruits have also been reported to possess hypoglycemic activity (Kamalakkannan and Prince, 2005). The present study was performed to understand the alterations of total muscarinic and muscarinic M1 receptor gene expression in the cerebral cortex during diabetes, insulin and *Aegle marmelose* leaf extract treated diabetic rats.

2. Materials and methods

2.1. Chemicals

Tris–HCl, pirenzepine and carbachol were purchased from Sigma Chemical Co., St. Louis, USA. Quinuclidinyl benzilate, L-[benzilic-4,4′-^{3}H], ([^{3}H]QNB)(specific activity 42 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, USA. Radioimmunoassay kits for insulin were purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India. Real-time PCR Taqman probe assays on demand were purchased from Applied Biosystems, Foster City, CA, USA.

2.2. Plant material

Fresh leaves of *Aegle marmelose* were collected from Cochin University area. The plants were taxonomically identified and authenticated by Mr. K.P. Joseph, Head of the Dept. of Botany (Retd), St. Peter's College, Kolenchery. The voucher specimens are deposited at the herbarium of the Centre for Neuroscience, Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala and the voucher number is MNCB No. 2.

2.3. Preparation of aqueous extract of Aegle marmelose leaves

Fresh leaves of *Aegle marmelose* were air dried in shade and powdered. 10 g of leaf powder was mixed with 100 ml of distilled water and stirred for 2 h. It was kept overnight at 4 °C. The supernatant was collected and evaporated to dryness followed by lyophylization in Yamato, Neocool, Japan lyophilizer. This was used as the crude leaf extract to study the antihyperglycemic effect in streptozotocin-induced diabetes.

2.4. Selection of animal

Male adult Wistar rats of 180–240 g body weight were used for all experiments. They were housed in separate cages under 12 h light and 12 h dark periods. Rats have free access to standard food and water ad libitum. The experimental protocol has been approved by our Institutional Ethics Committee.

2.5. Diabetes induction

Diabetes was induced in rats by intrafemoral injection of streptozotocin freshly dissolved in 0.1 M citrate buffer, pH 4.5, under anesthesia (Junod et al., 1969). Rats were anesthetize by in diethyl ether (Gillespie and Muir, 1967).Streptozotocin was given at a dose of 55 mg/kg body weight (Arison et al., 1967; Hohenegger and Rudas, 1971).

2.6. Experimental design

Twenty rats were divided into four equal groups as follows:

(a) *Group 1*. Control: rats of this group received single intrafemoral injection of 0.1 M citrate buffer, pH 4.5.
(b) *Group 2*. Diabetic: rats were made diabetic by single intrafemoral injection of streptozotocin freshly dissolved in 0.1 M citrate buffer, pH 4.5, under anesthesia.
(c) *Group 3*. Diabetic rats treated with insulin: this group received subcutaneous injections (1 unit/kg body weight) of insulin daily during the entire period of the experiment. The last injection was given 24 h before sacrificing the rats.
(d) *Group 4*. Diabetic rats treated with aqueous extract of *Aegle marmelose* leaves: aqueous extract of *Aegle marmelose* leaves was given orally to this group of diabetic rats in the dosage of 1 g/kg body weight (Ponnachan et al., 1993) at 24 h intervals during the entire period of the experiment.

The animals were considered as diabetic, if their blood glucose values were above 250 mg/dl on the third day after STZ injection. The treatment was started on the third after STZ injection and continued for 14 days.

2.7. Sacrifice of experimental rats

The animals were then sacrificed on 15th day by decapitation. The blood samples were collected and plasma was separated

by centrifugation. The tissue samples and plasma were kept at −70 °C until assay.

2.8. Estimation of blood glucose

Blood glucose was estimated by the spectrophotometric method using glucose oxidase–peroxidase reactions. Blood samples were collected from the tail vein at 0 h (Before the start of the experiment), 3rd day, 6th day, 10th day and 14th day and the glucose levels were estimated. Blood samples were collected 3 h after the administration of morning dose. The results were expressed in terms of milligram per deciliter of blood.

2.9. Estimation of circulating insulin by radioimmunoassay

Circulating insulin was measured according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

2.10. Total muscarinic and muscarinic M1 receptor binding studies in the cerebral cortex

[^{3}H]QNB binding assay in cerebral cortex (CC) was done according to the modified procedure of Yamamura and Synder (1981). Cerebral cortex was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris–HCl buffer, containing 1 mM EDTA, pH 7.4. The supernatant was then centrifuged at 30,000 × *g* for 30 min and the pellets were resuspended in appropriate volume of Tris–HCl–EDTA buffer.

Total muscarinic receptor binding parameter assays were done using different concentrations, i.e., 0.1–2.5 nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250 μl containing appropriate protein concentrations (200–250 μg). Non-specific binding was determined using 100 μM Atropine. Tubes were incubated at 22 °C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Muscarinic M1 receptor binding assays were done using different concentrations, i.e., 0.1–2.5 nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250 μl containing appropriate protein concentrations (200–250 μg). Non-specific binding was determined using 100 μM pirenzepine. Tubes were incubated at 22 °C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

2.11. Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein, which was read in a spectrophotometer at 660 nm.

2.12. Receptor data analysis

The receptor binding parameters were determined using Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on *X*-axis and bound/free on *Y*-axis using Sigma Plot Software (Version 2.0, Jandel GmbH, Erkrath, Germany). The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

2.13. Displacement curve analysis

The displacement data were analysed by nonlinear regression using Graph Pad PRISM^{TM} Software, Graph Pad Inc., San Diego, USA. The concentration of the competing drug that competes for half the specific binding was defined as EC_{50} which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Chen and Prusoff, 1973). The Hill slope was used to indicate a one or two-sited model of curve-fitting.

2.14. Analysis of gene expression by real-time PCR

RNA was isolated from the cerebral cortex of experimental rats using the Tri reagent (MRC, USA). Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μl contained 0.2 μg total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/μl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time PCR assays were performed in 96-well plates in ABI 7300 Real-Time PCR instrument (Applied Biosystems). The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 μl contained

25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer, and TaqMan probe for Muscarinic M1 receptor gene and endogenous control (β-actin) and 12.5 μl of Taqman 2× Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The following thermal cycling profile is used for 40 cycles: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 01 min.

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$) and the graph was plotted using log of ($2^{-\Delta\Delta CT}$).

2.15. Statistics

Statistical evaluations were done by ANOVA, expressed as mean ± S.E.M. using InStat (Ver.2.04a) computer programme.

3. Results

3.1. Effect of Aegle marmelose leaf extract on blood glucose level of experimental rats

Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration led to a significant increase ($p < 0.001$) in blood glucose level of diabetic group when compared to control group. Insulin treatment and aqueous extract of *Aegle marmelose* leaves treatment was able to significantly reduce ($p < 0.001$) the increased blood glucose level to near the control value when compared to diabetic group (Table 1).

3.2. Effect of Aegle marmelose leaf extract on plasma insulin level of experimental rats

There was a significant decrease in the plasma insulin level of the diabetic group when compared to control group ($p < 0.001$). Insulin treatment and aqueous extract of *Aegle marmelose* leaves supplementation for 14 days significantly increased ($p < 0.001$) the plasma insulin level to near control level when compared to diabetic group (Fig. 1).

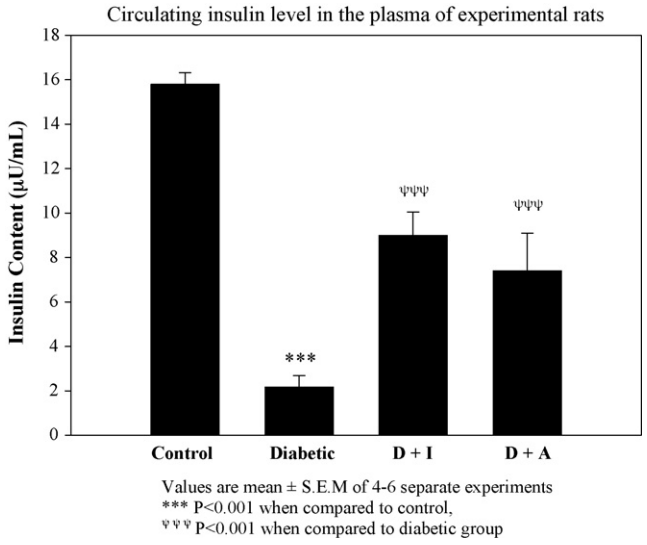

Fig. 1. Values are mean ± S.E.M. of four to six separate experiments. ***$p < 0.001$ when compared to control and $^{\psi\psi\psi}p < 0.001$ when compared to diabetic group.

3.3. Scatchard analysis for [^{3}H]QNB binding against atropine in the cerebral cortex of experimental rats

Scatchard analysis for cerebral cortex total muscarinic receptors showed that the B_{max} was decreased significantly ($p < 0.001$) in diabetic rats with a significant decrease ($p < 0.01$) in the K_d when compared to control group. In insulin treated diabetic rats B_{max} was significantly ($p < 0.001$) reversed to near control when compared to diabetic group. K_d also significantly ($p < 0.01$) reversed to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} ($p < 0.001$) and K_d ($p < 0.01$) to near control when compared to diabetic group (Table 2 and Fig. 2).

3.4. Displacement analysis of [^{3}H]QNB using atropine

The competition curve for atropine against [^{3}H]QNB fitted for one sited model in all groups. The log(EC_{50}) did not

Table 1
Blood glucose (mg/dl) level in experimental rats

Animal status	0 day (before STZ injection)	3rd day (initial)	6th day	10th day	14th day (final)
Control	85.3 ± 2.1	94.2 ± 1.4	90.2 ± 0.6	99.1 ± 1.8	98.1 ± 1.4
Diabetic	86.7 ± 1.7	267.3 ± 0.4	300.1 ± 0.6	310.7 ± 0.8	310.9 ± 1.0[a]
Diabetic + insulin (D + I)	84.1 ± 0.9	252.2 ± 0.9	303.4 ± 0.6	182.8 ± 1.3	138.0 ± 1.1[b,c]
Diabetic + *Aegle marmelose* (D + A)	85.3 ± 1.1	264.3 ± 0.3	303.8 ± 1.0	190.3 ± 1.8	118.0 ± 2.8[b,c]

Values are mean ± S.E.M. of four to six rats in each group.
[a] $p < 0.001$ when compared to control.
[b] $p < 0.001$ when compared to diabetic group.
[c] $p < 0.001$ when compared with initial reading.

Table 2
Scatchard analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	710.00 ± 5.77	0.90 ± 0.10
Diabetic	290.00 ± 5.77[b]	0.27 ± 0.02[a]
Diabetic + insulin treated	656.66 ± 29.06[d]	0.93 ± 0.06[c]
Diabetic + *Aegle marmelose* treated	680.00 ± 62.45[d]	1.15 ± 0.18[c]

Values are mean ± S.E.M. of four to six separate experiments.
[a] $p < 0.01$ when compared to diabetic group.
[b] $p < 0.001$ when compared to control.
[c] $p < 0.01$ when compared to diabetic group.
[d] $p < 0.001$ when compared to diabetic group.

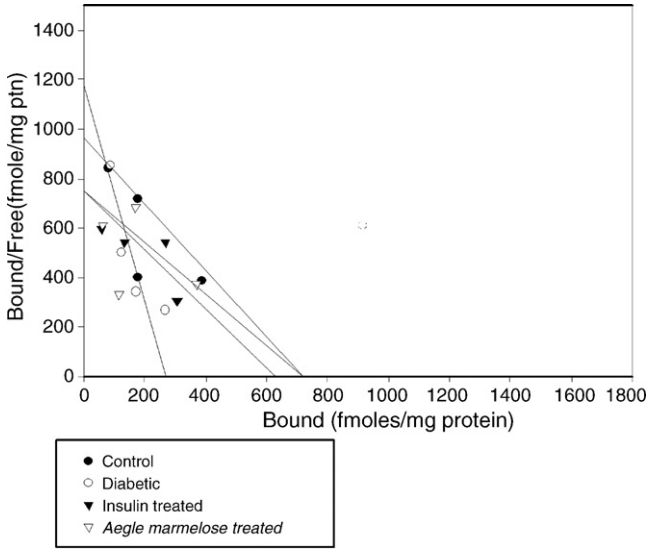

Fig. 2. Scatchard analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats. Graph showing Scatchard analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rat groups. Total muscarinic receptor binding parameter assays were done using different concentrations, i.e., 0.1–2.5 nM of [^{3}H]QNB. Non-specific binding was determined using 100 μM atropine.

change in all the experimental groups. The K_i decreased in diabetic condition and reversed to near control value in insulin and *Aegle marmelose* leaf extract treated diabetic rats (Table 3 and Fig. 3).

Table 3
Binding parameters of [^{3}H]QNB against atropine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats

Experimental group	Best-fit model	Log(EC_{50})	K_i	Hill slope
Control	One-site	−6.403	1.31×10^{-7}	0.9807
Diabetic	One-site	−6.552	9.35×10^{-8}	0.9731
Insulin treated diabetic	One-site	−6.485	1.09×10^{-7}	0.9918
Aegle marmelose treated diabetic	One-site	−6.136	2.43×10^{-7}	0.9981

Values are mean of four to six separate experiments.

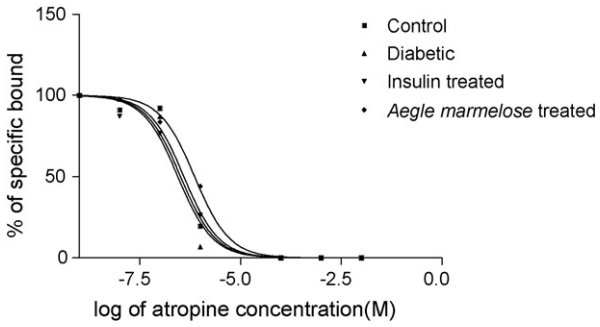

Fig. 3. Graph showing displacement analysis of [^{3}H]QNB binding against atropine in the cerebral cortex of control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rat groups. Competition studies were carried out with 1 nM [^{3}H]QNB in each tube with atropine concentrations varying from 10^{-9} to 10^{-4} M. Data were fitted with iterative nonlinear regression software (Prism, Graph Pad, San Diego, CA). K_i is the affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

3.5. Scatchard analysis for [^{3}H]QNB binding against pirenzepine in the cerebral cortex of experimental rats

Binding analysis of muscarinic M1 receptors was done using [^{3}H]QNB and M1 subtype specific antagonist pirenzepine. The B_{max} was decreased significantly ($p < 0.001$) in diabetic group when compared to control group. The K_d also decreased significantly when compared to control group ($p < 0.01$). In insulin treated diabetic rats B_{max} was significantly ($p < 0.001$) reversed to near control when compared to diabetic group. K_d also significantly ($p < 0.01$) reversed to near control when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} ($p < 0.001$) and K_d ($p < 0.01$) to near control value when compared to diabetic group (Table 4 and Fig. 4).

3.6. Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for pirenzepine against [^{3}H]QNB fitted for one sited model in all groups. The log(EC_{50}) did not change in all the experimental groups. The K_i decreased in diabetic condition and reversed to near control value in insulin and *Aegle marmelose* treated diabetic rats (Table 5 and Fig. 5).

Table 4
Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	2126.60 ± 14.50	2.62 ± 0.06
Diabetic	1433.30 ± 28.40[b]	1.35 ± 0.14[a]
Diabetic + insulin treated	2023.30 ± 14.50[d]	2.30 ± 0.18[c]
Diabetic + *Aegle marmelose* treated	2110.0 ± 30.50[d]	2.39 ± 0.25[c]

Values are mean ± S.E.M. of four to six separate experiments.
[a] $p < 0.01$ when compared to diabetic group.
[b] $p < 0.001$ when compared to control.
[c] $p < 0.01$ when compared to diabetic group.
[d] $p < 0.001$ when compared to diabetic group.

Table 5
Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats

Experimental group	Best-fit model	Log(EC_{50})	K_i	Hill slope
Control	One-site	−5.053	3.93×10^{-6}	−0.9904
Diabetic	One-site	−4.989	4.55×10^{-6}	−0.9928
Insulin treated diabetic	One-site	−5.205	2.77×10^{-6}	−0.9664
Aegle marmelose treated diabetic	One-site	−5.122	3.35×10^{-6}	−0.9914

Values are mean of four to six separate experiments.

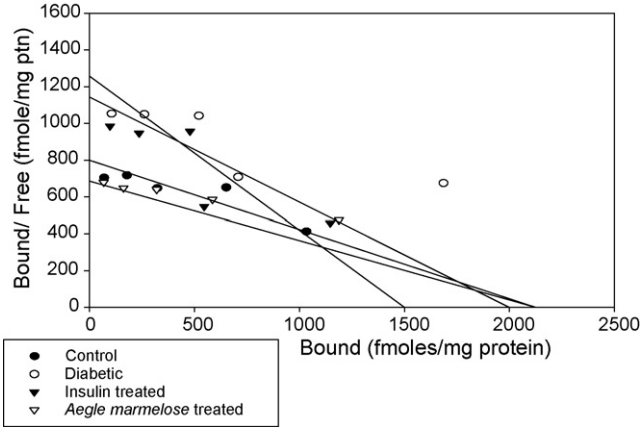

Fig. 4. Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of control, diabetic, diabetic + insulin treated and diabetic + *Aegle marmelose* treated group rats. Graph showing Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rat groups. Total muscarinic receptor binding parameter assays were done using different concentrations, i.e., 0.1–2.5 nM of [^{3}H]QNB. Non-specific binding was determined using 100 μM pirenzepine.

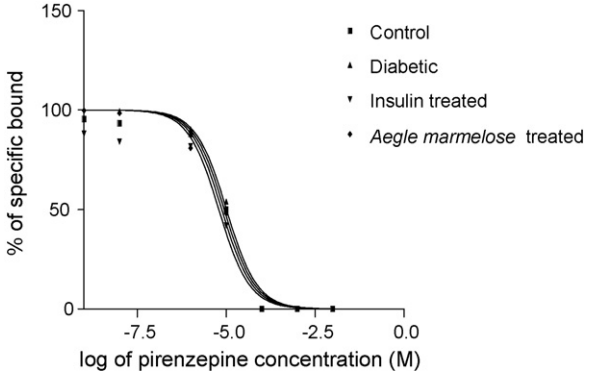

Fig. 5. Graph showing displacement analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rat groups. Competition studies were carried out with 1 nM [^{3}H]QNB in each tube with pirenzepine concentrations varying from 10^{-9} to 10^{-4} M. Data were fitted with iterative nonlinear regression software (Prism, Graph Pad, San Diego, CA). K_i is the affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table 6
Real Time amplification of mRNA from the cerebral cortex of Control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rats

Experimental group	RQ value
Control	0
Diabetic	-2.61 ± -0.15[a]
Diabetic + insulin treated	-0.09 ± -0.21[b]
Diabetic + *Aegle marmelose* treated	-0.23 ± 0.01[b]

Values are mean ± S.D. of four to six separate experiments. Relative quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the ΔΔCT method normalized with β-actin CT value as the internal control and control CT value as the calibrator.
[a] $p < 0.001$ when compared with control.
[b] $p < 0.001$ when compared with diabetic group.

3.7. Real-time PCR analysis of muscarinic M1 receptor mRNA from cerebral cortex of experimental rats

Real-time PCR analysis showed that the muscarinic M1 receptor mRNA expression was decreased significantly ($p < 0.001$) in diabetic rats while it reversed to control in insulin treated and *Aegle marmelose* treated diabetic rats when compared to diabetic group ($p < 0.001$) (Table 6 and Fig. 6).

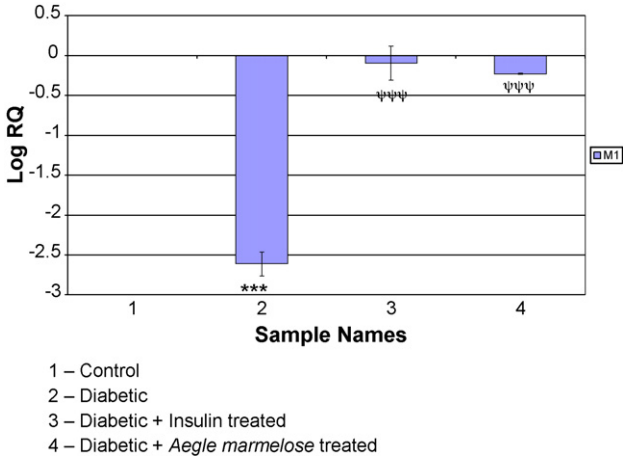

Fig. 6. Real-time amplification of mRNA from the cerebral cortex of control, diabetic, diabetic + insulin and diabetic + *Aegle marmelose* treated rats. (1) Control; (2) diabetic; (3) diabetic + insulin treated; (4) diabetic + *Aegle marmelose* treated.

4. Discussion

Diabetes mellitus is a heterogeneous metabolic disorder as old as mankind and its incidence is considered to be high (4–5%) all over the world (Pickup and William, 1997). *Aegle marmelose* leaf extract has previously been shown to be antihyperglycemic when orally administered to rats (Ponnachan et al., 1993; Das et al., 1996; Seema et al., 1996). Reports shows that the extract of the leaves of *Aegle marmelose* is non-toxic via intraperitoneal route in rats, at least up to maximum doses of 1000 mg/kg body weight acutely and 100 mg/kg body weight subacutely (Veerappan et al., 2007).

The present paper discusses the alterations of total muscarinic and muscarinic M1 receptors in the cerebral cortex during diabetes and the regulatory activity of *Aegle marmelose* on glucose homeostasis. Neurotransmitters are reported to have a key role in glucose-induced insulin secretion in the pancreatic islets and glucose homeostasis. In vitro and in vivo studies revealed the importance of muscarinic receptors in insulin secretion (Ahrén et al., 1999). Acetylcholine stimulated insulin secretion is inhibited by atropine, a general muscarinic antagonist, confirming the role of muscarinic receptors and cholinergic involvement in insulin secretion (Gilon and Henquin, 2001). The M1 muscarinic receptor is one of five known muscarinic subtypes in the cholinergic nervous system (Bonner et al., 1987; Hulme et al., 1990). M1 receptors are predominantly expressed in the forebrain, including the cerebral cortex, hippocampus and corpus striatum, where this sub-type contributes by 50–60% to the total of the muscarinic receptors (Hamilton et al., 1997; Gerber et al., 2001; Miyakawa et al., 2001). Our results showed that administration of *Aegle marmelose* leaf extract to STZ diabetic rats normalizes blood glucose level. The glucose lowering activity of *Aegle marmelose* leaf extract confirmed the previous reports (Ponnachan et al., 1993). Recent reports showed that Scopoletin (7-hydroxy-6-methoxy coumarin), isolated from the leaves of *Aegle marmelose* has potential to regulate hyperglycemia in levo-thyroxine-induced hyperthyroid rats (Panda and Kar, 2006). There was a significant decrease in the circulating insulin level of diabetic rats when compared to control group. The increase in insulin level in *Aegle marmelose* leaf extract treated diabetic rats attribute to the stimulation of the surviving beta cells by the extract, which in turn exerts an antihyperglycemic action. Reports are available to show that antidiabetic plants are known to increase circulating insulin levels (Lamela et al., 1985). Thus, it can be suggested that the *Aegle marmelose* leaf extract induce the synthesis and release of insulin thereby potentiating its effect. This data again confirmed the antihyperglycemic activity of *Aegle marmelose* leaf extract. Our results showed that Muscarinic M1 receptors are decreased in diabetic rats with decrease in affinity when compared to control group in the cerebral cortex. In insulin treated and *Aegle marmelose* leaf extracts treated diabetic rats, binding parameters were reversed to near control level.

Earlier studies from our laboratory have established the central neurotransmitter receptor subtypes functional regulation during diabetes, pancreatic regeneration, cell proliferation and insulin secretion (Paulose et al., 1988; Sudha and Paulose, 1988; Abraham and Paulose, 1999; Biju et al., 2001; Renuka et al., 2004; Mohanan et al., 2005a, b; Kaimal et al., 2007). Also it was established that muscarinic M1 receptors are involved in the glucose-induced insulin secretion (Renuka et al., 2006). Insulin receptors are widely distributed in the brain, with the highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex, cerebellum, and hippocampus (Havrankova et al., 1978; Van Houten et al., 1979). When the duration of noninsulin-dependent diabetes increases, both parasympathetic function (Nilsson et al., 1995) and β cell function deteriorate (Clauson et al., 1994). Hence, it might be speculated that the muscarinic binding parameters and gene expression changes observed during diabetes are directly associated with the decrease in β cell function. The mechanism by which insulin reverses the binding parameters and gene expression between insulin receptors and muscarinic receptors is through the direct interaction. In our study insulin and *Aegle marmelose* leaf extract reverses the changed binding parameters and gene expiration of muscarinic receptors in the cerebral cortex of the diabetic rats. This indicates the ability of the plant extract to increase muscarinic receptors in the cerebral cortex of the diabetic rats. Insulin receptors have been demonstrated to have influence on cholinergic neurons in the central nervous system (Araujo et al., 1989; Holdengreber et al., 1998) and on autonomic neurons in the periphery (Reinhardt et al., 1993; Karagiannis et al., 1997). Studies from our laboratory also concluded that muscarinic M1 receptors decrease in the hypothalamus, brainstem, and pancreatic islets of diabetic rats and the insulin treatment reversed these altered parameters near to control level (Gireesh et al., 2007). Our results showed that insulin treatment and also the *Aegle marmelose* leaf extract treatment reverses the decreased general and muscarinic M1 receptors near to control level in the cerebral cortex of the experimental rats. The central muscarinic M1 and M3 receptor subtypes functional balance regulate sympathetic and parasympathetic systems which in turn control the islet cell proliferation and glucose homeostasis (Renuka et al., 2004). Earlier work indicated that muscarinic brain networks can modulate vagus nerve functions such as brain regulation of glycogen synthesis in the liver, exocrine pancreatic secretion (Shimazu et al., 1976; Saito et al., 1994; Li et al., 2003; Wu et al., 2004). Intracerebral administration of muscarinic agonists also stimulates vagus nerve signaling a result that couples the activation of muscarinic brain network to increased vagus nerve activity in the periphery (Pavlov, 2006) and the acetylcholine mediates insulin release through vagal stimulation. Muscarinic receptors decreased significantly during diabetes in the cerebral cortex resulted in decreased vagal nerve stimulation and decreased acetylcholine mediated insulin secretion from the pancreas. *Aegle marmelose* reverses the decreased general and M1 muscarinic receptors in diabetes and increase the vagal nerve stimulation and thereby the insulin secretion. Thus our study suggest that decreased muscarinic and muscarinic M1 receptors during hyperglycemia and the regulatory activity of *Aegle marmelose* leaf extract on glucose homeostasis has clinical significance in management of diabetes.

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