Materials and Methods

CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals
Curcumin, Cholecalciferol (Vitamin D₃), dopamine, pirenzepine, atropine, 4-DAMP mustard (4-deoxy acetyl methyl piperidine mustard), ethylene diamine tetra acetic acid - EDTA, HEPES - [n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid], Streptozotocin, citric acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, collagenase type XI, bovine serum albumin fraction V and RPMI-1640 medium were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other reagents were of analytical grade purchased locally.

Radiochemicals
Quinuclidinylbenzilate, L-[Benzilic-4,4'-³H]-[4-³H] (Sp. Activity 42 Ci/mmol), [³H] Dopamine (Sp. activity- 45.1Ci/mmol) and 4-DAMP, [N-methyl-³H] (Sp. Activity 83 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, U.S.A.

Radioimmunoassay kit for insulin was purchased from Baba Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals
Tri-reagent kit was purchased from Sigma chemicals Co., St. Louis, MI, USA. ABI PRISM High capacity cDNA Archive kit, primers and Taqman probes for Real Time- PCR were purchased from Applied Biosystems, Foster City, CA, USA.
Confocal Dyes

Rat primary antibody for muscarinic M1 (Cat. No. 087k1395), M3 (Cat. No. 126k1205), α7 nicotinic acetylcholine receptor (Cat. No. 018k4811), acetylcholine esterase (Cat. No. 097k1431) and vesicular acetylcholine transporter (Cat. No. 077k4838) and FITC coated secondary antibody (Cat. No. No-AP307R) were purchased from Sigma Aldrich and Chemicon, USA.

ANIMALS

Adult male Wistar rats of 180-240g body weight purchased from Amrita Institute of Medical Sciences, Cochin and Kerala Agriculture University, Mannuthy were used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water ad libitum.

DIABETES INDUCTION

Diabetes was induced in rats by intrafemoral injection of streptozotocin (Sigma chemicals Co., St. Louis, MO, U.S.A.) freshly dissolved in citrate buffer pH 4.5 under anaesthesia (Junod et al., 1969). Streptozotocin was given at a dose of 55mg/Kg body weight (Hohenegger & Rudas, 1971; Arison et al., 1967).

DETERMINATION OF BLOOD GLUCOSE

The diabetic state of animals was assessed by measuring blood glucose concentrations at 72 hours after streptozotocin treatment. The rats with a blood sugar level above 250 mg/dl were selected as diabetic rats.
DETERMINATION OF ANTI-DIABETIC POTENTIAL OF CURCUMIN AND VITAMIN D₃

Animals used in this study were randomly divided into the following groups. Each group consisted of 6-8 animals.

a) Group 1: Control (given citrate buffer injection)
b) Group 2: Diabetic
c) Group 3: Diabetic rats treated with insulin
d) Group 4: Diabetic rats treated with Curcumin
e) Group 5: Diabetic rats treated with Vitamin D₃

The insulin treated diabetic group (Group 3) received subcutaneous injections (1Unit/kg body weight) of insulin daily during the entire period of the experiment. A mixture of both Lente and Plain insulin (Abbott India) were given for the better control of glucose (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats.

Curcumin was given orally to the 4th group of diabetic rats in the dosage of 60mg/Kg body weight suspension of curcumin orally at 24 hour intervals. Curcumin was suspended in 0.5% w/v sodium carboxymethylcellulose immediately before administration in constant volume of 5ml/kg body weight (Sharma et al., 2006). Cholecalciferol was given orally to the 5th group of diabetic rats in the dosage of 12 µg/Kg body weight dissolved in 0.3 ml of coconut oil (Rosanne et al., 2005). Blood samples were collected from the tail vein at 0 hours (Before the start of the experiment), 3rd, 6th, 10th and 14th day and the glucose levels were estimated. Blood samples were collected 3hrs after the administration of morning dose. Changes in the body weight of animals were monitored on 1st Day (before the start of the experiment), 7th and 15th day.
SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 15\textsuperscript{th} day by decapitation. The cerebral cortex, cerebellum, brain stem, corpus striatum, and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966) and the pancreas was dissected quickly over ice. Hippocampus was dissected according to the procedure of Heffner et al., (1980). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80\textdegree C until assay. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{(\text{GOD})} \text{Gluconic acid} + \text{H}_2\text{O}_2.
\]

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \xrightarrow{(\text{Peroxidase})} \text{Coloured complex} + \text{H}_2\text{O}
\]

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in (Shimadzu UV-1700 pharmaSPEC) spectrophotometer.
ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done 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 $[^{125}\text{I}]$ 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.

Assay Protocol

Standards, ranging from 0 to 200 µU/ml, insulin free serum and insulin antiserum (50µl each) were added together and the volume was made up to 250µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then $[^{125}\text{I}]$ insulin (50µl) was added and incubated at room temperature for 3 hours. The second antibody was added (50µl) along with 500µl of PEG. The tubes were then vortexed and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with %B/Bo on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/Bo was calculated as:

\[
\text{Corrected average count of standard or sample} \times 100
\]

\[
\text{Corrected average count of zero standard}
\]
Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

ESTIMATION OF CIRCULATING TRIIODOTHYRONINE (T3) BY RADIOIMMUNOASSAY

Principle of the assay
The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method was based on the competition of unlabelled endogenous T3 with $^{125}\text{I}$ T3 for the limited binding sites on the antibody (Ab1) made specifically for T3. The antibody was in the form of a complex with second antibody (Ab2). At the end of incubation, the T3 (Ag) bound to the antibody-second antibody complex (Ag-Ab1-Ab2) and free T3 was separated by the addition of PEG. The amount bound to the antibody complex in the assay tubes were compared with values of known T3 standards and the T3 concentration in the samples were calculated.

Assay Protocol
Standards, ranging from 0.15 to 2.5 ng, T3 free serum, $^{125}\text{I}$ T3 and antiserum complex were added together and the volume was made up to 275 µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated at 37°C for 45 minutes. The PEG was added to all tubes and they were centrifuged at 1500xg for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with %B/B₀ on the Y-axis and T3 concentration (ng /ml) on the X-axis of a log-logit graph. %B/B₀ was calculated as:
Corrected average count of standard or sample

\[ \frac{\text{Corrected average count of zero standard}}{100} \]

T3 concentrations in the samples were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

**BEHAVIOURAL STUDIES**

**Y-Maze Test**

The Y-maze was made of grey wood, covered with black paper and consisted of three arms with an angle of 120 degrees between each of the arms. Each arm was 8 cm width \( \times \) 30 cm length \( \times \) 15 cm height. The three identical arms were randomly designated: Start arm, in which the rat started to explore (always open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial; and the other arm (always open). The maze was placed in a separate room with enough light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze.

The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10 minutes duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 hour ITI (Ma et al., 2007), the second trial (retention) was conducted, during which all three arms were accessible and novelty vs familiarity was analyzed through comparing behavior in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 minutes. The time spent in each arm was analyzed. Data was expressed as percentage of performance in all three arms during the five minutes of test (Akwa et al., 2001, Jobin, et al., 2010).
Rotarod Test:
Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on a revolving rod (Dunham & Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 15 days of treatment in all groups of rats.

Grid Walk Test
Deficits in descending motor control were examined by assessing the ability to navigate across a 1 m long runway with irregularly assigned gaps (0.5–5 cm) between round metal bars. Crossing this runway requires that animals accurately place their limbs on the bars. In baseline training and postoperative testing, every animal had to cross the grid for at least three times. The number of footfalls (errors) was counted in each crossing for 3 minute and a mean error rate was calculated (Z'Graggen et al., 1998).

Narrow Beam Test
The narrow beam test was performed according to the descriptions of Haydn and Jasmine (1975). A rectangular 1.2-cm wide beam, 1.05m long and elevated 30 cm from the ground was used for the study. After training, normal rats were able to traverse the horizontal beams with less than three footfalls. When occasionally their feet slipped off the beam, they were retrieved and repositioned precisely. The time the rats could remain balanced on the beam was counted.
MUSCARINIC AND DOPAMINE RECEPTOR BINDING STUDIES USING [$^3$H] RADIOLIGANDS

Binding studies in the Brain regions

Total muscarinic, muscarinic M1 and M3 receptor binding studies

[$^3$H] QNB and [$^3$H] DAMP binding assay in cerebral cortex, cerebellum, brain stem, hippocampus, corpus striatum and pancreas were done according to the modified procedure of Yamamura & Snyder (1981). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA (pH 7.4). The supernatant was then centrifuged at 30,000g for 30 minutes and the pellets were resuspended in appropriate volume of Tris-HCl-EDTA buffer.

Total muscarinic and muscarinic M1 receptor binding parameter assays were done using [$^3$H] QNB (0.1-2.5nM) and M3 receptor using [$^3$H] DAMP (0.01-5nM) in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). The non-specific binding was determined using 100µM atropine for total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP mustard for muscarinic M3 receptor. Total incubation volume of 250 µl contains 200-250µg protein concentrations. Tubes were incubated at 22°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM 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.
**Total Dopamine receptor binding studies.**

Dopamine DA receptor assay was done using $[^3]H$ DA as per Madras et al., (1988) and Hamblin & Creese, (1982). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 4mM MgCl$_2$, 1.5 mM CaCl$_2$, pH 7.4 and centrifuged at 38,000xg for 30min. at 4°C. The pellet was washed twice by homogenization and centrifuged twice at 38,000 g for 30min. at 4°C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-1.5nM of $[^3]H$ DA in 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 1mM MgCl$_2$, 2 mM CaCl$_2$, 120mM NaCl, 5mM KCl, pH 7.4 in a total incubation volume of 250µl containing 200-300 µg of proteins. Specific binding was determined using 100µM unlabelled dopamine. Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

**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 660nm.
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ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data were analysed according to 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. 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.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS

Isolation of RNA

RNA was isolated from the brain regions and pancreas of control and experimental rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 100 µl of chloroform was added to the homogenate, mixed vigorously for 15 seconds kept in the RT for 10-15 minutes and was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and
absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance 260 = 42 µg.

REAL-TIME POLYMERASE CHAIN REACTION

cDNA synthesis
Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2ml microfuge tubes. The reaction mixture of 20 µl contained 0.2µg total RNA, 10 X RT buffer, 25 X dNTP mixture, 10 X random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 minutes and 37 °C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express software version (3.0).

Real-time PCR assays
Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase “polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe, designed by Applied Biosystems. All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5’ end and a quencher (Minor Groove Binding Protein - MGB) at the 3’ end. The Real-Time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

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 PCR
analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of muscarinic M1, M3, α7 nicotinic acetylcholine, dopamine D1, dopamine D2, insulin, Vitamin D receptors, acetylcholine esterase, choline acetyl transferase, GLUT3, GLUT2, super oxide dismutase, phospholipase C, PDX1 and CREB. Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5 µl of TaqMan 2X Universal PCR Master Mix was taken and the volume was made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50ºC -- 2 minutes ---- Activation
95ºC -- 10 minutes ---- Initial Denaturation
95ºC -- 15 seconds ---- Denaturation 40 cycles
50ºC -- 30 seconds --- Annealing
60ºC -- 1 minutes --- Final Extension

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 (ΔCT = CT_{Target} – CT_{β-actin}). It was further normalized with the control (ΔΔCT = ΔCT – CT_{Control}). The fold change in expression was then obtained (2^{-ΔΔCT}).
IMMUNOHISTOCHEMISTRY OF MUSCARINIC M1, M3 AND α7 NICOTINIC ACETYLCHOLINE RECEPTOR IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rat were transcardially perfused with PBS, pH 7.4, followed by 4% paraformaldehyde in PBS (Chen et al., 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH 7.0. 10 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.01% Triton X-100) for 20 min. Brain slices were incubated overnight at 4°C with either rat primary antibody for muscarinic M1, M3 and α7 nicotinic acetylcholine receptor, diluted in PBST at 1: 500 dilution) (polyclonal or monoclonal). After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of FITC. The sections were observed and photographed using confocal imaging system (Leica SP 5). The specificity of the immunohistochemical procedure is validated by negative controls to ensure that the labelling method accurately, identified the antibody bound to the specific muscarinic M1, M3 and α7 nicotinic acetylcholine receptor in the brain regions. Expressions of muscarinic M1, M3 and α7 nicotinic acetylcholine receptor were analysed using pixel intensity method. The given mean pixel value is the net value which is deducted from the negative control pixel value (Peeyush et al., 2010).
IMMUNOCYTOCHEMISTRY OF MUSCARINIC M1, M3 RECEPTORS, ACETYLCHOLINE ESTERASE AND VESICULAR ACETYLCHOLINE TRANSPORTER EXPRESSION IN THE PANCREAS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Pancreatic islets were isolated from control and experimental rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers et al., 1985) with the following composition: 137 mM Choline chloride, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, 14.3 mM KHCO$_3$ and 10 mM HEPES. The pancreas was aseptically transferred to a sterile glass vial containing 2.0 ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300 rpm/minute). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% was assessed by Trypan Blue. The islets were seeded in culture wells and allowed to adhere to the plate. The islets were rinsed with PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0., for 30 minutes on ice. After fixation, the islets were washed thrice with blocking buffer containing 0.1 M phosphate buffer, pH 7.0., 0.1% Triton X and 10% BSA. Then the islets were incubated with primary antibody for muscarinic M1, M3 receptors, acetylcholine esterase and vesicular acetylcholine transporter, diluted in PBST at 1: 1000 dilution), prepared in blocking buffer with 1% serum and incubated overnight at 4°C. After the incubation, the islets were washed thrice with blocking buffer. Then the islets were incubated with secondary antibody tagged with FITC (No: AB7130F, Chemicon, diluted in PBST at 1: 1000 dilution) diluted in blocking buffer with 1% serum and incubated at room temperature in dark for two hours. After incubation the islets were rinsed with blocking buffer and were observed and
photographed using confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure is validated by negative controls to ensure that the labelling method accurately identifies the antibody bound to the specific muscarinic M1, M3 receptors, acetylcholine esterase and vesicular acetylcholine transporter in the pancreatic islets. Expressions of muscarinic M1, M3 receptors, acetylcholine esterase and vesicular acetylcholine transporter were analysed using pixel intensity method. The given mean pixel value is the net value which is deducted from the negative control pixel value (Peeyush et al., 2010).

STATISTICS
Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.