Protection of Testicular Dysfunctions by MTEC, a Formulated Herbal Drug, in Streptozotocin Induced Diabetic Rat

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Single injection of streptozotocin (STZ) resulted diabetes mellitus which was reflected here by the levels of fasting blood glucose and serum insulin. Moreover, this experimental diabetes also resulted testicular dysfunctions evaluated by count, viability and motility of sperm as well as by the activities of key enzymes for androgen synthesis. Diabetes induced testicular oxidative stress has been indicated here by the monitoring of testicular peroxidase and catalase activities as well as by quantification of TBARS and CD of testis. Testicular glucose was increased and Leydig cell nuclear area was decreased in STZ induced diabetes. Treatment of herbal formulated drug named as MTEC consist of aqueous-methanol extract of Musa paradisiaca, Tamarindus indica, Eugenia jambolana and Coccinia indica to streptozotocin induced diabetic rat at the ratio of 2 : 2 : 1 : 1 at the dose of 60 mg/d for two times a day for 14 d resulted a significant protection in fasting blood glucose and serum insulin levels (p<0.05) along with correction of testicular above parameters towards the control level (p<0.05). This herbal formulated drug has no general toxic effects on the body weight, as well as on the activities of serum glutamine and pyruvate transaminases in serum. The results support the validity of this herbal drug for the management of testicular disorders noted in diabetic state.

Key words diabetes; insulin; testosterone; sperm count; androgenic key enzyme; oxidative stress; herbal drug

Diabetes is associated with declining sexual function in both male19 and female20 individuals. In case of female, diabetic symptoms consistent with autonomic neuropathy associated with decreased subjective sexual arousal.3 In recent study Erol et al., noted that women with type 2 diabetes experienced higher prevalence rates of sexual dysfunction when compared to non diabetic.4 Approximately 80% of diabetic patients complained loss of libido, 60% had diminished clitoral sensitivity, 50% experienced organic dysfunction, 40% experienced vaginal discomfort, and 40% experienced vaginal dryness.5 Type 1 diabetic women experienced higher prevalence of reduced vasocongestion and reduced vaginal lubrication to erotic stimuli.6 Thus these clinical studies suggest that both type 1 and type 2 impede the sexual arousal responses in women.

Male reproductive alterations have been widely reported in model animals and human with diabetes.7 Streptozotocin induced diabetes in male rats resulted atrophy of sex organ, changes in histoarchitecture of ventral prostate,8 diminution in sperm count,9 along with low levels in plasma gonadotrophins9,10 and testosterone.11 The copulatory behavior of diabetic rat has been collapsed12 along with low fertility in induced normal mating.12 In human, diabetes is associated with erectile dysfunction13,14 along with loss of libido1,5 and abnormal morphology of sperm, low plasma levels of gonadotrophins and testosterone.15

Though there are several reports about dysfunctions of the male reproductive activity in diabetic state but the data are confusing, and the exact role that insulin plays in the regulation of male reproductive function is still unclear.

In traditional practice, medicinal plants are used in many countries to control diabetes mellitus. The National Centre for Complementary and Alternative Medicine, established in 1998 by the United States Government where development of herbal medicine is one of the important subjects of study.6,17 Moreover the herbal drug is of first choice at present because plant drugs are to be less toxic and more free from side effect than synthetic one.18 M. paradisiaca is a tree like herb belonging to the Musaceae family and different parts of the plant have medicinal value.19,20 T. indica Linn is tree type of plant belonging to the Caesalpinia family was used as a traditional medicine for the management of diabetes mellitus.21 E. Jambolana is used as traditional medicine for the management of diabetes.22 This plant is found all over India and belongs to the Myrtaceae family. Jambu bark cure hemorrhages, burning sensation, dysentery, diarrhea, diabetes, excessive thirst, dyspepsia, cough and asthma and is used in preparation of astringent decoction for gargles and washes.20 Seeds are used for the treatment of diabetes.20 C. indica is under Cucurbitaceae family and has medicinal importance as observed earlier by others.22

In our previous work we have reported the antidiabetic effect of some of these plant parts in separate and in composite manner but not these four plant parts in a single formula.23–26 In this experiment an approach has been considered to find out the composite effect of these four plant parts in a single formula on the basis of previous idea that composite extracts of plants are used as the drug of choice rather than individual and many of these shown promising effects.23 In this study an attempt has been taken to strengthen the idea about the testicular disorders noted in diabetes and to evaluate the antidiabetic potency and the remedial effect of the herbal formulation known as MTEC on testicular dysfunction in diabetic state. Here we have considered the aqueous methanol extract of these four plant parts in a specific ratio in streptozotocin induced diabetic male rat as these solvent extract has a greater potency for this purpose. For this

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formula, the above ratio has been selected on the basis of the trial and error where this ratio execute the most promising results in this purpose.

MATERIALS AND METHODS

Chemicals Streptozotocin was obtained from Spectrochem Pvt. Ltd., a chemical company (India). Insulin enzyme linked immunosorbant assay (ELISA) kit purchased from Boehringer Mannheim Diagnostic, Mannheim (Germany). Testosterone kit was purchase from IBL–Germany.

Plant Materials and Formulation of MTEC The root of Musa paradisiaca (M. paradisiaca), seed of Tamarindus indica (T. indica), seed of Eugenia jambolana (E. jambolana) and leaves of Coccinia indica (C. indica) were collected from local area in the month of June and the materials were identified by taxonomist of Botany Department, Vidyasagar University, Midnapore. The voucher specimens having HPCH No-1, 6, 7, 8 were deposited in the said Department.

Small pieces of fresh root of M. paradisiaca, seed of T. indica, seed of E. jambolana and leaves of C. indica were dried in an incubator for 2 d at 40°C, crushed separately in an electric grinder and then powdered. Out of this powder, 50 g of each was suspended in 250 ml of aqueous–methanol (2 : 3) mixture separately in separate container and kept in incubator at 37°C for 36 h. The slurry was stirred intermittently for 2 h and left overnight. The mixture was then filtered and filtrate was dried by low pressure and residue was collected. An antidiabetic drug was formulated named as MTEC with these four plants extract using the above solvent residue of M. paradisiaca, T. indica, E. jambolana, C. indica at the ratio of 2 : 2 : 1 : 1. When required the residue was suspended in olive oil in a fixed dose and used for treatment.

Selection of Animal and Animal Care Twenty-four matured normoglycemic Wistar strain male albino rats of 3 month of age weighing about 150±10 g were taken for this experiment. Animals were acclimated for a period of 15 d in our laboratory condition prior to the experiment. Rats were housed at an ambient temperature of 25±2°C with 12 h light : 12 h dark cycle. Rats have free access to standard food and water ad libitum. The principles of laboratory animal care (NIH 1985) and instruction given by our institutional ethical committee were followed through out the experimentation.

Induction of Diabetes Mellitus Fasting rats for 24 h were subjected to single intramuscular injection of streptozotocin (STZ) at the dose of 4 mg/0.1 ml of citrate buffer/100 g body weight/rat that produce type 1 diabetes (having fasting blood glucose level more than 250 mg/dl but less than 350 mg/dl) after 24 h of STZ injection. Sixteen rats were made diabetic in this way.

Animal Treatment Twenty-four rats were divided into three groups equally as follows and duration of experiment was of 14 d.

Group I: (Control group): Rats of this group received single intramuscular injection of citrate buffer (0.1 ml/100 g body weight/rat) as vehicle treatment.

Group II: (Diabetic group): Rats of this group were made diabetic by single intramuscular injection of streptozotocin at the dose of 4 mg/0.1 ml citrate buffer/100 g body weight/rat.

Group III: (MTEC co-administered group): The diabetic rats of this group were forcefully fed by gavage method with MTEC at the dose of 60 mg/0.5 ml olive oil/100 g body weight/rat/twice daily after 24 h of STZ injection. First dose was given in the morning 8.00 a.m. in fasting condition and second dose at the evening 6.00 p.m., 4 h after the food intake. This experiment was continued for 14 d.

Animals of control (group I) and diabetic groups (group II) were subjected to forceful feeding of 0.5 ml of olive oil/100 g body weight/d for 14 d at the time of MTEC co-administration to the animals of group III to keep all the animals in same experimental condition.

From the starting day of extract co-administered to diabetic rats of group III fasting blood glucose levels in all the groups were measured by single touch glucometer at the interval of 2 d throughout the experiment.

On 16th day of experiment, all the animals were sacrificed by decapitation after recording the final body weight, blood was collected from dorsal aorta and serum was separated by centrifugation at 3000 g for 5 min for the assay of insulin followed by ELISA technique. Epididymis and testes were dissected out and one testis was stored at −20°C for biochemical analysis and other testis was used for histological study. Cauda epididymis of each animal was stored at 37°C in buffer and used for epididymal sperm count and sperm viability.

Testing of Fasting Blood Glucose Level At the time of grouping of the animals, fasting blood glucose (FBG) level was measured. At 2 d interval, FBG was further recorded from all the animals of all groups. Blood was collected from the tip of the tail vein and FBG level was measured by single touch glucometer.

Serum Insulin Level Serum insulin was measured by enzyme linked immunosorbant assay (ELISA) using the kit (Boehringer Mannheim Diagnostic, Mannheim, Germany). The intra assay variation was 4.9%. As the samples were run at a time, so there was no inter assay variation. The insulin level in serum was expressed in μIU/ml.

Serum Testosterone Level Serum levels of testosterone were measured using the testosterone kit from IBL–Germany according to the standard protocol supplied by that company. In this solid phase-conjugated assay an alkaline phosphatase conjugated hormone was used. The company supplied the chromogen and stop solution. The optical density of standard and unknown was measured by using the selective filter at 450 nm and differentiating filter at 630 nm. The intra-assay variation was 5.2%. There was no inter assay variation as all the samples were assayed at a time.

Sperm Viability Sperm viability was perform by the eosin nigrosin staining. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead sperm were differentiated from unstained live sperm, and there numbers were recorded.

Estimation of Testicular Δ4,3β-Hydroxysteroid Dehydrogenase (Δ4,3β-HSD) and 17β-Hydroxysteroid Dehydrogenase (17β-HSD) Activities Testicular Δ4,3β-HSD activity was measured according to the method of Talalay.
One testis from each animal was homogenized carefully at 4 °C in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration 100 mg/ml homogenizing mixture. This mixture was centrifuged at 10000 g for 30 min at 4 °C. The supernatant (1 ml) was mixed with 100 μM sodium pyrophosphate buffer (pH 8.9), 40 μl ethanol containing 30 μg dehydro epiandrosterone and 960 μl of 25% w/v BSA, bringing the incubation mixture to a total of 3 ml. Enzyme activity was measured after addition of 0.5 μM NAD to the tissue supernatant mixture in a spectrophotometric cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

For the measurement of testicular 17β-HSD activity, another 1 ml supernatant from the same homogenizing mixture was added to 440 μM sodium pyrophosphate buffer (pH 10.2), 40 μl ethanol containing 0.3 μM testosterone and 960 μl of 25% w/v BSA, bringing the incubation mixture to a total of 3 ml. Enzyme activity was measured according to the method of Jarabak et al.,32 after addition of 1.1 μM NADP to the tissue supernatant mixture in a spectrophotometric cuvette at 340 nm against a blank (without NADP). One unit of enzyme activity was equivalent to a change in absorbance of 0.001/min at 340 nm.

Testicular Glucose Testicular tissue was homogenized in phosphate buffer saline at the concentration of 50 mg/ml and centrifuged at 6000 rpm and the supernatant was collected. This supernatant was used for the measurement of glucose spectrophotometrically33 and the value was expressed in mg/100 mg of tissue.

Biochemical Assay of Testicular Catalase The activity of catalase of the testicular tissues was measured biochemically.34 For the evaluation of catalase activity testis of each animal were homogenized separately in 0.05 M Tris–HCl buffer solution (pH-7.0) at the tissue concentration of 50 mg/ml. These homogenized samples were centrifuged at 10000 g at 4 °C for 10 min. In spectrophotometric cuvette, 0.5 ml of 0.00035 M H2O2 and 2.5 ml of distilled water were mixed and reading of absorbance was noted at 240 nm. Supernatant of testicular sample was added at a volume of 40 μl and the subsequent six readings were noted at 30 s interval.

Biochemical Assay of Glutathione Peroxidase The glutathione peroxidase activity was measured biochemically.35 Liver and skeletal muscle tissues were homogenized in ice cold of 0.1 M phosphate buffer saline (pH 7.4) at the tissue concentration of 50 mg/ml followed by centrifugation at 8000 g for 15 min. The reaction mixture contents 2.525 ml of 0.1 M Tris–HCl buffer (pH-7.2), 75 μl of 0.04 ml GSH (reduced glutathione), 100 μl of 0.1 M nicotinamide adenine dinucleotide phosphate (NADPH) and 100 μl of glutathione reductase (0.24 unit). One hundred micro liters of supernatant was added to 2.8 ml of reactive mixture and incubated at 25 °C for 5 min. The reaction was initiated by adding 100 μl of 0.75 mm/l H2O2, and then its absorbance was measured at 340 nm for 5 min. The activity was expressed as nmol NADPH oxidized/mg tissue/min using a molar extinction coefficient of 6.22×104 (nmol/l/cm).

Estimation of Lipid Peroxidation from the Concentration of Thiobarbituric Acid Reactive Substance (TBARS) and Conjugated Dienes (CD) Testis was homogenized separately at the tissue concentration of 50 mg/ml in 0.1 M of ice-cold phosphate buffer (pH-7.4) and centrifuged at 10000 g at 4 °C for 5 min. Each supernatant was used for the estimation of TBARS and CD. For the measurement of TBARS, the homogenate mixture of 0.5 ml was mixed with 0.5 ml of normal saline (0.9 g% NaCl) and 2 ml of TBA–TCA mixture (0.392 g thiobarbituric acid in 75 ml of 0.25 N HCl with 15 g trichloroacetic acid. The volume of the mixture was made up to 100 ml by 95% ethanol) and boiled at 100 °C for 10 min. This mixture was then cooled at room temperature and centrifuged at 4000 g for 10 min. The whole supernatant was taken in spectrophotometer cuvette and read at 535 nm.36

Quantification of the CD was performed by a standard method.37 The lipids were extracted with chloroform–methanol (2 : 1) followed by centrifugation at 1000 g for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance was noted at 233 nm to measure the amount of hydro peroxide formed and expressed in nmol/mg of tissue using the formulae.

\[
\text{nmol/mg of tissue} = \frac{\text{OD} \times 10^4}{1.5 \times \text{mg of tissue used in assay}}
\]

Biochemical Assay of Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT) For the measurement GOT and GPT activities in serum, kits were used supplied by Crest Biosystems, Gitanjali, Dr. Antonio Do Rego Bagh, Alto Santacruz, Bambolim Complex (Goa, India). The activities of these enzymes were expressed as unit.38

Quantification of Germ Cells at Stage VII in Seminiferous Epithelial Cycle The hematoxylin–eosin stained slides were scanned under light microscope. Quantification of different generation of germ cell at stage VII was performed according to the method of Leblond and Clermont.39 The cells present in this stage are spermatogonia-A (SgA), preleptotene spermatocytes (pLSc), mid-pachytene spermatocyte (mPSc), step 7 spermatids (7Sd) and 19 spermatids (19Sd). The different nuclei of the germ cells (except step19 spermatids, which can not be precisely counted) were counted at 20 round tubular cross-sections in each rat. All the nuclear count of germ cells were corrected for differences in nuclear diameter by the formulae of Abercrombie,40 True count = (Crude count×section thickness)/(section thickness+diame-
ter of germ cell), and tubular shrinkage by the Sertoli cell correction factor.41

Seminiferous Tubular Diameter (STD) and Leydig Cell Nuclear Area (LCNA) Using software in computer, we have measured the leydig cell nuclear area (LCNA) and seminiferous tubular diameter (STD) in all of these three groups from stained histological sections

Statistical Analysis Analysis of Variance (ANOVA) followed by multiple two-tail ‘t’ test was used for statistical analysis of collected data.42 Differences were considered significantly at \( p<0.05 \).

RESULTS

Body Weight and Organo-Somatic Indices
growth of STZ induced diabetic animal was decreased significantly in comparison to control group. Co-administration of MTEC to the diabetic animals for two times/d for 14 d resulted a significant recovery in body growth towards the control though significantly lower than the control level (Table 1).

**Fasting Blood Glucose Level** Induction of diabetes by STZ resulted a significant elevation in plasma glucose in comparison to control and this level is more than 180 mg/dl (Fig. 1). After the co-administration of MTEC to the diabetic animals for 14 d resulted a significant recovery of the parameter towards the control (Fig. 1).

**Testicular $D_5,3bb-HSD$ and $17bb-HSD$ Activities and Testicular Glucose** There was significant diminution in the activities of $D_5,3bb-HSD$ and $17bb-HSD$ in STZ induced diabetic rat when compare to control. The activities of above enzymes in testis were recovered significantly after co-administration of MTEC to diabetic animal for two times/d for 14 d (Fig. 2). Testicular glucose was increased significantly in diabetic group when compared to control. A significant recovery was noted in above parameters after co-administration of MTEC to the diabetic group (Fig. 2).

**Serum Insulin and Serum Testosterone** Serum levels of insulin and testosterone both were decreased significantly after STZ induced diabetic state in respect to control. Co-administration of MTEC to the diabetic animal resulted a significant corrective effect on these parameters towards the control (Fig. 3).

**Sperm Count, Viability of Sperm and Histology of Testis** Epididymal sperm count and sperm viability both were decreased significantly in STZ induced diabetic state when compared to matched control group. Co-administration of MTEC, the formulated herbal drug, resulted the significant recovery in the above parameters towards the control level (Table 2). Numbers of $Sg A$, pLSc, mPSc, 7Sd all were decreased significantly at stage VII of seminiferous epithelial cycle in STZ induced diabetic state in comparison to control. The count of the above germ cells at stage VII was protected towards the control level though significant differ from control.

**Table 1.** Effect MTEC on Body Weight and Testiculo-Somatic, Prostato-Somatic and Seminal Vesiculo-Somatic Indices in Streptozotocin-Induced Diabetic Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Testiculo-somatic index (g%)</th>
<th>Seminal vesiculo-somatic index (g%)</th>
<th>Prosteto-somatic index (g%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>152.66±4.7a</td>
<td>160.33±3.9b</td>
<td>1.4578±0.27a</td>
<td>0.4261±0.024a</td>
</tr>
<tr>
<td>Diabetic</td>
<td>156.33±5.2a</td>
<td>140.61±2.3b</td>
<td>1.1932±0.26a</td>
<td>0.1995±0.037b</td>
</tr>
<tr>
<td>MTEC co-administered</td>
<td>154.75±5.4a</td>
<td>153.0±3.7c</td>
<td>1.3523±0.21c</td>
<td>0.3689±0.25c</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E.M., n=8. ANOVA followed by multiple comparison two tail ‘t’ test. Values with different superscript (a,b,c) in each vertical column differ from each other significantly (p<0.05).

![Fig. 1. Effect of MTEC on Fasting Blood Glucose Level in Streptozotocin Induced Diabetic Male Albino Rat](image1)

![Fig. 2. Protective Effect of MTEC on Testicular $D_5,3bb-HSD$, $17bb-HSD$ Activities and Testicular Glucose in Streptozotocin Induced Diabetic Male Albino Rat](image2)

![Fig. 3. Changes in Serum Insulin and Serum Testosterone Levels in Streptozotocin Induced Diabetic Male Albino Rat after Co-administration of MTEC](image3)
trol after co-administration of MTEC (Table 2). In diabetic group, there was a significant diminution in STD and LCNA in respect to control. After co-administration of MTEC to the diabetic rat, a significant elevation in both the above parameters was noted (Table 2).

Activities of Catalase and Peroxidase Activities of catalase and peroxidase in testis were decreased significantly in diabetic group in respect to control group. After treatment of MTEC, activities of the above mentioned parameters in testis were significantly recovered towards the control when compare to diabetic group (Fig. 4).

Conjugated Diene and Thiobarbituric Acid Reactive Substance Level Testicular CD and TBARS levels were increased significantly in diabetic group in respect to control. There was a significant recovery in above mentioned parameters in testis after co-administration of MTEC in diabetic animals in comparison to STZ induced diabetic group (Fig. 5).

Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT) Levels SGOT and SGPT levels were increased significantly in diabetic group in respect to control group. After co-administration of MTEC, a significant recovery was noted in above parameters toward the control levels (Fig. 6).

DISCUSSION

Diabetes mellitus, a multifaceted multiorgan disorder declared as a disease of complication, is prevalent globally and has been projected to become one of the world’s main dis-
tory. This duration of treatment is the minimal and effective that exhibits the corrective results which have been observed by duration dependent study. After streptozotocin injection, fasting blood glucose level was increased and serum insulin levels was decreased which are in line of our previous work.23) After the co-administration of MTEC to the diabetic rat, there was a significant recovery in fasting blood glucose level and serum insulin which may be due to regeneration of β cells as proposed earlier by us.25) Streptozotocin induced diabetes resulted significant diminution in prosta-to-somatic, seminal vesiculo-somatic and testiculo-somatic indices which may be due to low serum level of testosterone, as testosterone is the prime regulator of normal growth of these organs.45) After administration of this formulated drug, the above organo-somatic indices were recovered towards the control level, which may be due to elevation in serum tes-
tosterone. This elevation in serum testosterone after co-adminis-
tration of this formulated drug may be due to elevation in the activities of androgenic key enzymes i.e., \( \Delta^5,3\beta\)-HSD and 17β-HSD60) as well as by the recovery of serum insulin as insulin has positive role in testicular testosterone synthesis.47,48) Another possibility for the corrective role of this formulated drug on testis may be the protection in testicular oxidative stress noted here in diabetic state by the measurement of testicular catalase and peroxidase activities, important scaveng-
ing enzymes of reactive oxygen species,49) and quantification of testicular TBARS and CD, the products of free radical.50)

The sperm count, which was decreased in diabetes, may be due to inhibition in spermatogenesis as reflected here by the quantification of different generation of germ cells at stage VII of spermatogenic cycle, an important reflector of holistic approach of spermatogenesis.51) Another possibility of low sperm count may be the effect of testicular oxidative stress in diabetic state as reported by other.22) Sperm motility and viability are also affected by oxidative damaging effect of free radicals.53) Diabetes induced testicular disorders have been focused here by the high testicular glucose level from this work but the work is in progress and the molecular action would be expressed from the subsequent work in this line.

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