ORIGINAL ARTICLE

Hyperglycemia-induced alteration in reproductive profile and its amelioration by the polyherbal formulation MTEC (modified) in streptozotocin-induced diabetic albino rats

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Abstract This study investigated oxidative stress-mediated alterations in the reproductive profile of streptozotocin-induced diabetic albino rats and their amelioration by the polyherbal formulation MTEC (modified), constituted with n-hexane fractions of hydromethanol extracts of Musa paradisiaca roots, Tamarindus indica seeds, Eugenia jambolana seeds, and Coccinia indica leaves in a specific ratio. We noted a change in serum insulin levels and a modulation in carbohydrate metabolic parameters, i.e. increased fasting blood glucose level, elevated glucose-6-phosphatase and lactate dehydrogenase activities, along with a diminution of hexokinase and glucose-6-phosphate dehydrogenase activities in liver, skeletal muscle, and cardiac muscle, which confirmed the streptozotocin-induced diabetic state. Glycogen levels were decreased in the liver and skeletal tissues. A diminution in reproductive function in the diabetic state was reflected here by significant low values in reproductive organo-somatic indices, sperm count, and motility, along with serum testosterone, but high levels of testicular cholesterol and seminal vesicular fructose. A diminution in activity of the principal antioxidative enzymes along with increased levels of free radical products in the primary and accessory sex organs, as well as in sperm pellets, also confirmed the development of oxidative stress in the male reproductive organs. An increased expression of testicular proapoptotic Bax gene in diabetes also supports the elevation of testicular germ cell apoptosis in the diabetic state. Oral administration of the herbal drug MTEC (modified) at a dose of 10 mg/0.5 mL 2% Tween 80 per 100 g body weight twice daily to the diabetic rats for 28 days significantly corrected the...
Diabetic therapeutic efficacy of polyherbal MTEC

Introduction

Diabetes is a very common endocrine disorder that poses a serious challenge to healthcare worldwide. Diabetes mellitus is a multidimensional disease known as a "syndrome." It is an endocrine and chronic metabolic disorder characterized by a chronic hyperglycemic state resulting from a malfunction of insulin secretion and/or insulin action caused by impaired regulation of carbohydrate, lipids, and protein homeostasis. It is now the seventh greatest killer of mankind due to its high prevalence and mortality.

Diabetes mellitus has a detrimental effect on sexual function, especially in male individuals. Alterations in male reproductive functions develop in diabetes as it modulates the endocrine control of spermatogenesis by imposing an oxidative stress that results in decreased libido, delayed sexual maturation, and infertility with poor semen quality. Stages of spermatogenesis including Stages IX–XI are also affected, and a significant increase in the number of degenerated germ cells at various stages of development has also been noted in diabetes mellitus, with decreased levels of serum gonadotropins and testosterone.

Although the exact mode of regulation of male reproductive function by insulin is still unclear, there is a positive relationship between free testosterone and fasting insulin levels in diabetic men independent of age, obesity, and body fat distribution. Prospective studies have shown that low testosterone levels predict the development of diabetes in men.

Reactive oxygen species (ROS) play an important role in the normal function and pathophysiology of sperm. Elevated levels of ROS are related to peroxidative damage of the spermatozoal membrane, rich in docosahexaenoic acid and susceptible to ROS, which leads to an increase in permeability of the sperm membrane, morphologic abnormalities, and impaired fertility. The ultimate results are severe oligospermia, asthenospermia, and male infertility.

The relationship between human aortic endothelial cell cultures with a high glucose concentration and apoptotic phenomena has been proved by in vitro study. Increased levels of expression of Bax gene in human diabetic retinas and in the kidney cortex in diabetic animal models correlate with high levels of glucose.

Apoptosis of testicular germ cells balances the number of germ cells during the normal process of spermatogenesis. However, in response to death signals, for example hormonal deprivation and pathologic stress, the mitochondrial pathway of germ cell apoptosis is modulated by proapoptotic Bax protein by increasing the release of cytochrome C and/or endoplasmic reticulum-mediated Ca\(^{2+}\), which leads to testicular damage.

Despite progress in the management of diabetes by synthetic drugs, the search for improved, safe, and natural antidiabetic agents is still ongoing and has also been recommended by the World Health Organization. In line with this, we have formulated MTEC (modified), which is a composite of the solvent fractions of specific plant parts. Musa paradisiaca Lam. (family—Musaceae) is a tree-like herb distributed throughout India and Malaysia. Different parts of the plant are claimed to have medicinal values. We have also reported the antidiabetic activity of M. paradisiaca root. Tamarindus indica Linn. (family—Caesalpiniaaceae) and Eugenia jambolana (E. jambolana) Linn. (family—Myrtaceae) are both tree-type plants whose seeds are used as a traditional medicine for the management of diabetes mellitus, as reported by us and others. Coccinia indica Wight & Am (family—Cucurbitaceae) is a herb widely distributed all through India, and its medicinal values have been supported by our findings.

These four plants are used in folk medicinal practice to treat diabetes mellitus in remote villages in West Bengal, India. We have already reported the antidiabetic activity of "MTEC" (which takes its name from the first letters of the scientific name of each plant used here), a polyherbal formulation we prepared by taking the crude aqueous extracts of these four plants in a ratio of 2:2:1:1 (w/w), respectively. Following our previous success, this study was designed to develop a more potent form of MTEC, using n-hexane fractions of hydroalcohol extracts of these four plant parts in a ratio of 2:2:1:1 (w/w), respectively, that would have a low dose of efficacy for the management of diabetes and diabetes-induced, oxidative stress-related, apoptosis-mediated testicular dysfunction.

In drug development, one of the important steps is isolation of the more effective biomolecule(s) present in the plant parts. To this end, effective fractions have been identified that will provide a clue for the pharmaceutical industry in formulating an effective herbal drug against diabetes. The philosophy of using a polyherbal formulation...
over a monoherbal drug has been adopted here, as previ-
ously reported by us.18 These fractions are more effective
for this purpose than other fractions that have been studied
using a dose- and duration-dependent trial-and-error
method.

Materials and methods

Plant material and herbal formulation of MTEC

Fresh roots, seeds, and leaves of M. paradisiaca, T. indica,
E. jambolana, and C. indica were collected from rural areas
of Paschim Medinipur district, West Bengal, India during
May to July. Preliminary identification of the plants was
made by a taxonomist in the Botany Department, Vidyasa-
gar University, Midnapur, India. A voucher specimen (HPCH
No. 7, 1, 6, 8) was deposited in the Department of Botany at
Vidyasagar University.

After collection, the plant parts were separated and
washed thoroughly first with tap water and then with
deonized water. The plant parts were finally completely
dried in an incubator at 37°C. About 6.2 kg of dried roots,
4.0 kg of dried leaves, and 4.8 kg of each of the dried seeds
were collected from 11.6 kg of fresh roots, 20 kg of fresh
leaves, and 5.3 kg and 6 kg, respectively, of fresh seeds.
The dried plant parts were pulverized separately with an
industrial electrical grinder. Then, using a 20-L percolator,
maceration was carried out separately in hydromethanol
solvent (H2O:MeOH 40:60, v/v, 250 mL of solvent being used
for 50 g of each plant part), with intermittent stirring for
the first 2 hours and left without stirring for 36 hours at
37°C to avoid any degradation or deactivation of the active
component(s).

The extraction process for each plant part was continued
for the 3 days following the previous process, and the final
extracts were collected separately on the 4th day. These
extracts were then filtered through first a cotton filter and then
a No. 1 Whatman filter paper in the form of slurry. The
hydromethanol filtrate was evaporated under reduced pres-
sure (10–200 mbar) using a rotatory evaporator instrument
(Hahn-Shin HS-2000NS; Pung-Dong, Gyeonggido, Korea) at
38°C to produce final fractions of 9.07 g, 5.30 g, 3.09 g,
and 4 g, respectively, collected as solvent-free
residues into amber-colored glass containers.16 The poly-
herbal antidiabetic drug MTEC was then formulated using
these solvent fractionates of the above four plant parts in a
ratio of 2:2:1:1 (by weight), respectively.

Test animals

Normoglycemic male albino Wistar rats aged 2 months and
weighing about 130 ± 10 g were used in these experiments.
The animals were housed in cages at an ambient temperature
of 25 ± 2°C with a 12-hour light/12-hour dark cycle, and
acclimated to these conditions for 15 days before being used
in the trials. Rats were fed food and water ad libitum. The
principles of laboratory animal care and instruction given by
our Institutional Ethics Committee (VU/IAEC/ BioMed/08/
2008-2009)16 were followed throughout the experiment.

Induction of diabetes mellitus, animal grouping and
treatment

Initially, 30 normoglycemic rats were selected for this
study, 24 of which were subjected to 18 hours of fasting
and induction of diabetes by a single intramuscular injec-
tion of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO,
USA) at a dose of 3.5 mg/100 g body weight in citrate
buffer (pH 4.5) as standardized by previous work in our
laboratory.16,18

Diabetes was confirmed by estimation of fasting blood
glucose (FBG) level after an interval of 24 hours and then on
the 7th day after the day of injection to investigate the
stability of the diabetic condition. Rats with an FBG greater
than 250 mg/dL but less than 350 mg/dL were included in
this investigation. Out of 24 rats, 20 rats were found to be
diabetic, and of these, 18 diabetic rats and six normogly-
cemic rats were included in the study. The experiment
lasted 28 days. The initial body weight of all 24 rats was
recorded, and they were then divided into the following
equally sized groups:

- **Group I (control group):** Rats received a single intra-
muscular injection of citrate buffer (0.1 mL/100 g of
body weight for each rat).
- **Group II (untreated diabetic group):** Rats were made
diabetic by a single intramuscular injection of STZ at a
dose of 3.5 mg/0.1 mL of citrate buffer/100 g body
weight for each rat.
- **Group III (MTEC-treated diabetic group):** Diabetic rats
were treated with MTEC by gavage in the fasting state
at a dose of 10 mg/0.5 mL of 2% TWEEN 80 per 100 g of
body weight for each rat twice a day on and from the
7th day for the next 28 days.
- **Group IV (glibenclamide-treated diabetic group):** Dia-
abetic rats were treated with standard drug glibencla-
midine by gavage in the fasting condition at a dose of 6
mg/0.5 mL of 2% TWEEN 80 per 100 g of body weight
for each rat twice a day on and from the 7th day for the
next 28 days.

Groups I and II were subjected to forced feeding of
0.5 mL of 2% TWEEN 80/100 g of body weight twice a day for
28 days at the time when the animals in Groups III and IV
were being treated with MTEC or glibenclamide, to keep all
the animals under the same experimental conditions.

Every day, the first oral dose of the above-mentioned
formulation was given at 7 AM, one hour before supplying
the animal feed in the morning at 8:00 AM. A second oral
dose was administered at 7 PM, two hours after cleaning
the feed box in the afternoon at 5:00 PM. Feed was supplied
again to the animals 1 hour after the second oral adminis-
tration of the formulation and feed box was separated from
the cage on next day at 5 AM.
From the day of MTEC or glibenclamide treatment to rats in Group III or IV, the FBG of all the groups was monitored using a single-touch glucometer (Ascensia Entrust; Bayer, GmbH, Germany) at 2-day intervals. On the 29th day of the experiment (considering the day of treatment with MTEC or glibenclamide to be the 1st day), all the animals were sacrificed by decapitation after recording the final body weight and organ wet weight. Blood was collected from the dorsal aorta by syringe, and the serum was separated by centrifugation at 3000 × g for 5 minutes to assess insulin level and metabolic toxicity parameters. Packed cell pellets were used to measure glycated hemoglobin (HbA1c).

The testes, cauda epididymides, and seminal vesicles were removed and weighed to measure the organo-somatic index (organ weight × 100/weight). The liver, skeletal muscle, and cardiac muscles were dissected out, wet weights were noted separately, and the samples were stored at −20°C for biochemical analysis of the carbohydrate metabolic enzymes. One testis of each animal was fixed in Bouin’s fixative for histologic study. Using a suspension medium, sperm were washed out from the cauda epididymis to monitor sperm count and motility, and for sperm pellet preparation. The remaining testis and the epididymis of each animal were used for western blot analysis and biochemical study, as well as for assessment of the activities of the antioxidative enzymes catalase, peroxidase, superoxide dismutase (SOD), and glutathione S-transferase (GST), and for quantification of levels of conjugated diene (CD) and thiobarbituric acid reactive substance (TBARS) in the target tissues. Seminal vesicular fluid was used for fructose quantification.

Measurement of hyperglycemic profile

FBG was measured using the single-touch glucometer by collecting blood from the tip of the tail of all experimental and control animals in all groups on starting day of the experiment and thereafter at every 2 days interval throughout the experiment. Serum insulin level was measured using a solid phase-conjugated sandwich enzyme-linked immunosorbent assay (ELISA) kit for rats (EZRMI-13K; Millipore, St. Charles, Missouri, USA). Hba1c level and hepatic and skeletal tissue glycogen content were measured following our laboratory standard protocol. The activities of the principal carbohydrate metabolic enzymes hexokinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase in the hepatic, skeletal, and cardiac tissues were assayed following the established methods used in our laboratory.

Measurement of reproductive profile

Epididymal sperm count and sperm motility
Spermatozoa were collected from an equal length of the cauda of the excised epididymis of each rat in all the groups by flushing the cauda with the same volume (10 mL) of a suspension medium containing 140 mM/L of NaCl, 0.3 mM/L of KCl, 0.8 mM/L of Na2HPO4, 0.2 mM/L of KH2PO4, and 1.5 mM/L of D-glucose (pH adjusted to 7.3 by adding 0.1 M/L of NaOH) (Merck, Mumbai, India). The collected sample was centrifuged at 100 × g for 2 minutes, and the precipitate was resuspended in 10 mL of fresh medium. The number of spermatozoa in a 100 µL suspension was counted using a hemocytometer slide. The sperm count was expressed as the number of sperm per milliliter of suspension. The number of motile spermatozoa in 100 µL of suspension was counted under the microscope after placing the sample on a glass slide and covering it with a cover slip. The result was expressed as a percentage after counting 100 sperm in each field.

Quantification of seminal vesicular fructose level
Seminal vesicular homogenate was prepared at a tissue concentration of 50 mg/mL to measure its fructose level. The supernatant was deproteinized by adding 50 µL of ZnSO4 and 50 µL of NaOH to make a total dilution of seminal plasma of 1:16. This was followed by centrifugation at 400 × g for 15 minutes. For fructose measurement, 200 µL of this clear supernatant was used. The optical density of the standard and sample were measured against a blank at 470 nm. The concentration of fructose was obtained by plotting the value on a standard curve, with the value expressed as µM/mg of tissue.

Estimation of testicular cholesterol level
Testicular cholesterol was estimated using the kit supplied by Angstrom Biotech Pvt. Ltd. (Vododara, India). Cholesterol ester present in the testicular homogenate is acted on by cholesterol esterase to release fatty acid and cholesterol, which is oxidized by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide, together with 4-aminoantipyrine and a phenolic compound in the presence of peroxidase, forms the colored complex. The intensity of the color is proportionate to the total cholesterol level, which was measured at 505 nm.

Assay of serum testosterone by ELISA
Serum testosterone level was measured using a solid phase-conjugated assay by noting the optical density of standard and unknown samples using a 480 nm selective filter and a 650 nm differentiating filter. The testosterone kit was supplied by Lilac Medicare (P) Ltd (Mumbai, India). The intra-assay variation was 5.2%. Interassay variation was not applicable as all samples were assayed at the same time.

Assessment of antioxidative enzyme and lipid peroxidation end-product profile

Biochemical assessment of catalase, peroxidase, SOD, and GST activities
The activities of catalase, peroxidase, SOD, and GST of the testis, cauda epididymis, and sperm pellet were measured biochemically following our standard laboratory method.

Estimation of end-products of lipid peroxidation (CD and TBARS)
The sample tissues were homogenized separately at a tissue concentration of 50 mg/mL in 0.1 M of ice-cold phosphate buffer (pH 7.4), and the homogenates were centrifuged separately at 10,000 × g at 4°C for 5 minutes. Each supernatant was used for the spectrophotometrical
Acute toxicity study and biochemical estimation of metabolic toxicity markers glutamic oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT)

To establish the safety profile of the formulation, an acute toxicity study was carried out as per established guidelines. Healthy, adult, normoglycemic male albino Wistar rats, starved overnight, were divided into four groups of six rats each—control, 50-mg-treated, 100-mg-treated, and 300-mg-treated. They were orally fed with the 2% Tween 80 and MTEC (modified) in increasing dose levels of 50, 100, and 300 mg/100 g body weight in 2% Tween 80. The rats were kept under supervision continuously for 2 hours to record behavioral, neurologic, and autonomic profiles, and after a period of 24 and 72 hours for any death. Specif
cikits (Span Diagnostics Ltd., Surat, India) were used to measure serum GOT and GPT activity as a marker of metabolic toxicity, with the results expressed as relative units. 

Western blot analysis of testicular tissues

Frozen testicular tissues were ground in ice-cold radioimmunoprecipitation buffer (1 mL/g of tissue), and 100 μL of protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) was added. This was frozen at −20°C, thawed, and centrifuged at 15,000 × g for 20 minutes at 4°C. The supernatant was then collected. Protein content was determined using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) following the Bradford method. Protein samples (100 μg/well) along with prestained protein marker (5 μL) were electrophoresed on 14% resolving gel for 2 hours at 80 V. One gel was transferred to Coomassie Brilliant Blue stain to check the protein profile, and the second gel, electrophoresed under identical conditions, was used for western blot analysis. Resolved protein from the gel was transferred to the PVDF membrane (0.45 μm; Millipore Corporation, Billerica, MA, USA) using a semi-dry electrophotoblotting system (Hoefer, Inc. Holliston, MA, USA) at 150 mA for 1.5 hours. The blot was rinsed once in Tris buffer saline (TBS; pH 7.5), and blocking was carried out by 5% non-fat dry milk in Tris buffer saline with Tween 20 (TBST; pH 7.5) overnight. Washed blot was incubated with primary mouse monoclonal antibody (sc 7480; Santa Cruz Biotechnology, Inc. Heidelberg, Germany) for Bax-α (21 kDa; 1:200 dilution in TBST) overnight at 4°C. Incubation with rabbit polyclonal antibody anti β-actin (A2066; 42kDa; 1:6000 dilution in TBST; Sigma-Aldrich, St. Louis, MO, USA) served as an internal control to monitor equal loading of protein. Incubated blot was washed twice with TBST (1% Tween 20) and once with TBST (0.5% Tween 20) for 5 minutes each. Blots were incubated with horseradish peroxidase (HRP) linked goat anti-mouse, anti-rabbit secondary antibody diluted (1:2000) in TBST (0.5% Tween 20) at room temperature for 2 hours followed by washing with TBST and Tris buffer saline (TBS), and the labeled protein bands were visualized using the DAB system (Bangalore Genei, Bangalore, India). Densitometric analysis was performed with LabWorks image analysis software (Ver 4.0, UVP Inc., Upland, CA, USA).

Histological study

The testicular tissue was dissected out, slices being prepared from the same region of all the groups and fixed in Bouin’s fixative. These were subjected to paraffin embedding after a proper dehydration process, followed by section-cutting in a rotary microtome (4 μm thick; Semi-automated Rotary Microtome Leica RM2245; Leica Microsystems GmbH, Wetzlar, Germany) and hematoxylin and eosin staining for microscopic examination in accordance with laboratory procedures. Histologic examination was carried out on stained sections using AVerCap (Version 2.5; AVerMedia Technologies Inc., Fremont, CA, USA) and DeWinter Caliper Pro software (Version 3.0; DeWinter Optical Inc., West Patel Nagar, Delhi, India).

Statistical analysis

All experimental trials were replicated three times. Analysis of variance followed by a multiple comparison two-tail t test was used to compare the groups. Differences were considered significant at p < 0.05.

Table 1 Correction in the mean fasting blood glucose level in hyperglycemic streptozotocin (STZ)-induced diabetic rats after treatment with the polyherbal formulation MTEC (modified) or glibenclamide.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean fasting blood glucose level (mmol/L)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 Daya</td>
</tr>
<tr>
<td>Group I (Control group)</td>
<td>3.47 ± 0.15</td>
</tr>
<tr>
<td>Group II (Untreated diabetic group)</td>
<td>3.25 ± 0.12</td>
</tr>
<tr>
<td>Group III (MTEC treated diabetic group)</td>
<td>3.31 ± 0.12</td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated diabetic group)</td>
<td>3.36 ± 0.14</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error, n = 6. Analysis of variance followed by a multiple comparison two-tail t test.

a Significant difference (p < 0.05) compared with the control group on the same day.

**Significant difference (p < 0.05) after treatment in each group in comparison with the control group.

a STZ injections were given to the diabetic, MTEC-treated, and glibenclamide-treated groups.

b Treatment was started in the MTEC-treated and glibenclamide-treated groups.
Results

Hyperglycemic profile assessment

A significant ($p < 0.05$) elevation in FBG was noted in untreated diabetic animals, the blood glucose level being greater than 250 mg/dL, compared with nondiabetic control rats (Table 1). Treatment of diabetic animals with MTEC (modified) or glibenclamide for 28 days resulted in a significant ($p < 0.05$) lowering of FBG level (Table 1).

Serum insulin level, the glycogen content of hepatic tissue and skeletal muscle, and the activities of hepatic, skeletal and cardiac muscle hexokinase and glucose-6-phosphate dehydrogenase were significantly decreased ($p < 0.05$) in the untreated diabetic group in comparison with the control group animals. Administration of the polyherbal formulation MTEC (modified) or glibenclamide to diabetic animals resulted in a significant ($p < 0.05$) recovery of serum insulin level and glycogen content, along with restoration of the activities of the carbohydrate metabolic enzymes towards control levels. The levels of HbA1c, in blood, and the activities of glucose-6-phosphatase and lactate dehydrogenase in the above-mentioned target tissues, in untreated diabetic rats were higher than, and the said values in diabetic rats were decreased significantly ($p < 0.05$) towards the control levels after treatment with MTEC or glibenclamide (Table 2, Fig. 1).

Reproductive profile screening

Biomarkers for monitoring semen quality, for example sperm count and sperm motility, were significantly decreased ($p < 0.05$) in untreated diabetic rats in comparison to the control rats, which were increased significantly ($p < 0.05$) toward control levels in MTEC (modified)- or glibenclamide-treated diabetic rats (Table 3).

Significantly decreased ($p < 0.05$) levels of serum testosterone along with increased testicular cholesterol and seminal vesicular fructose levels were found in the diabetic group with respect to controls, and were significantly ($p < 0.05$) rectified toward control levels after treatment with MTEC (modified) or glibenclamide (Table 3).

Oxidative stress markers profile assessment

Activities of catalase, peroxidase, SOD, and GST in the testis, cauda epididymis, and sperm pellet were significantly ($p < 0.05$) decreased in the diabetic group in comparison to the control group. When STZ-treated diabetic rats were treated with the polyherbal formulation MTEC (modified) or glibenclamide, the levels of these parameters were restored significantly ($p < 0.05$) toward the control levels (Fig. 2). Levels of end-products of lipid peroxidation, i.e. CD and TBARS, in the reproductive tissues and sperm pellet were significantly increased ($p < 0.05$) in the diabetic group when compared to the control group. There was a significant ($p < 0.05$) recovery in the levels of the above parameters in the target tissues after treatment with MTEC (modified) or glibenclamide to the animals in the diabetic group (Fig. 2).

Western blot analysis

Western blot densitometric analysis of protein expression of the apoptosis biomarker Bax-α also indicated an increased level of testicular apoptosis in the diabetic rats when compared to control rats. A significant ($p < 0.05$) remedial activity of MTEC (modified) or glibenclamide on protein expression was noticed in the target tissues after treatment of the animals in the diabetic group (Fig. 3).

Histological study

Degeneration in the germ cell layers in the seminiferous tubules was observed in the diabetic rats with respect to the control group rats, and was corrected to some extent in qualitative terms after treatment of the diabetic rats with MTEC (modified) formulation or glibenclamide (Fig. 4).

Analysis of body weight and reproductive organo-somatic indices

The body weight and reproductive organo-somatic indices of the diabetic animals were decreased in comparison with the control group. Treatment of diabetic rats with MTEC (modified) or glibenclamide twice a day for 28 days corrected the decrease in body weight and organo-somatic indices (Table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycated hemoglobin level (mmol/mol)</th>
<th>Glycogen (μg of glucose/mg of tissue)</th>
<th>Serum insulin level (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Muscle</td>
<td></td>
</tr>
<tr>
<td>Group I (Control group)</td>
<td>2.95 ± 0.02</td>
<td>11.0 ± 0.51</td>
<td>7.11 ± 0.33</td>
</tr>
<tr>
<td>Group II (Untreated diabetic group)</td>
<td>29.07 ± 1.39</td>
<td>4.90 ± 0.22</td>
<td>2.82 ± 0.13</td>
</tr>
<tr>
<td>Group III (MTEC treated diabetic group)</td>
<td>11.04 ± 0.60</td>
<td>6.11 ± 0.28</td>
<td>4.28 ± 0.19</td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated diabetic group)</td>
<td>11.70 ± 0.65</td>
<td>7.19 ± 0.38</td>
<td>4.51 ± 0.21</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error, n = 6. Analysis of variance followed by a multiple comparison two-tail t test.
*Significant difference ($p < 0.05$) compared with the control group.
**Significant difference ($p < 0.05$) after treatment in each group compared with the control group.
***Significant difference ($p < 0.05$) after treatment in each group compared with the control group.
Acute toxicity study and biochemical estimation of metabolic toxicity markers

MTEC (modified) at its maximum dose level of 300 mg/100 g of body weight did not produce any significant changes in autonomic, behavioral, or neurologic parameters. Acute toxicity studies revealed the nontoxic nature of the MTEC (modified) formulation.

Activities of serum GOT and GPT were found to be unaltered ($p > 0.05$) in all the groups treated with the

**Figure 1** Amelioration in the activities of carbohydrate metabolic enzymes (hexokinase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, and lactate dehydrogenase) in liver and skeletal and cardiac muscle after treatment with MTEC (modified) or glibenclamide in streptozotocin-induced diabetic rats. Analysis of variance followed by multiple comparison two-tail $t$ test. Bars represent mean ± standard error ($n = 6$ for each group). Bars with different letters (a, b, c) differ significantly from each other ($p < 0.05$).

Acute toxicity study and biochemical estimation of metabolic toxicity markers

MTEC (modified) at its maximum dose level of 300 mg/100 g of body weight did not produce any significant changes in autonomic, behavioral, or neurologic parameters. Acute toxicity studies revealed the nontoxic nature of the MTEC (modified) formulation.

Activities of serum GOT and GPT were found to be unaltered ($p > 0.05$) in all the groups treated with the

**Table 3** Correction in reproductive profile (sperm count, sperm motility, and seminal plasma fructose, testicular cholesterol, and serum testosterone levels) after treatment with the polyherbal formulation MTEC (modified) or glibenclamide in streptozotocin-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sperm count (Million/mL epididymal fluid)</th>
<th>Sperm motility (%)</th>
<th>Seminal vesicular fructose level (µM/mg of tissue)</th>
<th>Testicular cholesterol level (mg/dL)</th>
<th>Serum testosterone level (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control group)</td>
<td>20.77 ± 1.01</td>
<td>76.16 ± 3.41</td>
<td>2.61 ± 0.12</td>
<td>35.78 ± 1.61</td>
<td>0.066 ± 0.0030</td>
</tr>
<tr>
<td>Group II (Untreated diabetic group)</td>
<td>8.21 ± 0.37*</td>
<td>31.81 ± 1.41*</td>
<td>4.73 ± 0.23*</td>
<td>47.23 ± 2.17*</td>
<td>0.002 ± 0.0001*</td>
</tr>
<tr>
<td>Group III (MTEC treated diabetic group)</td>
<td>14.33 ± 0.65**</td>
<td>61.11 ± 2.73**</td>
<td>3.27 ± 0.15**</td>
<td>38.13 ± 1.74</td>
<td>0.012 ± 0.0007**</td>
</tr>
<tr>
<td>Group IV (Glibenclamide treated diabetic group)</td>
<td>13.81 ± 0.62**</td>
<td>64.22 ± 2.87**</td>
<td>3.01 ± 0.14**</td>
<td>36.07 ± 1.61</td>
<td>0.013 ± 0.0007**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error, $n = 6$. Analysis of variance followed by a multiple comparison two-tail $t$ test.

*Significant difference ($p < 0.05$) compared with the control group.

**Significant difference ($p < 0.05$) after treatment in each group compared with the control group.
polyherbal formulation MTEC (modified) in comparison to the control group (Table 5).

Discussion

This study was designed to develop the more potent and more effective MTEC (modified) compared to our previous formulated polyherbal drug—MTEC—which was the aqueous methanol extract of the equivalent plant parts, for the management of STZ-induced diabetes. STZ causes a selective destruction of insulin-secreting pancreatic beta-cells by ROS-mediated oxidative damage, resulting in insulin-dependent diabetes mellitus. The elevation of FBG and HbA1c, along with the diminution in levels of liver and skeletal muscle glycogen in STZ-induced diabetic rats, due to low levels of serum insulin, are consistent with our earlier findings. Diabetes induced by hypoinsulinemia has been further increased by the diminution in the activities of hexokinase and glucose-6-phosphate dehydrogenase in liver, skeletal muscle, and cardiac muscle as these enzymes are under the positive regulation of insulin.
impairment of spermatogenesis in diabetic rats reduces testosterone levels by suppressing the activities of the antioxidative enzymes and the elevated levels of TBARS and CD in reproductive tissues, which results in impaired spermatogenesis. The result also may be due to low levels of serum insulin and testosterone.

Auto-oxidation of glucose and nonenzymatic glycation decreases the production of hydrogen peroxide and the superoxide radical (O$_2^\cdot$-) radicals, which decrease the activities of the principal antioxidative enzymes SOD, peroxidase, catalase, and GST in the diabetic condition. The decreased enzyme activity of SOD further enhances the production of O$_2^\cdot$- in the target tissues. Diminution in the activity of peroxidase results in decreased protection against ROS. In the highly pro-oxidant diabetic environment, the reduced enzyme activity of catalase might be due to this elevated level of O$_2^\cdot$. The reduced activity of the GST enzyme in hyperglycemic conditions also fails to detoxify endogenous compounds such as peroxidized lipids. In diabetes, hypoinsulinemia increases the activity of the enzyme fatty acyl coenzyme A oxidase, which initiates the beta-oxidation of fatty acids, resulting in lipid peroxidation. Further, the induction of oxidative stress in the testis and sperm in the diabetic state might be due to the high level of uncoupler protein synthesis in sperm, or due to a hypoxic state as diabetes results in HbA$_1c$ formation that interferes with oxygen delivery at the target tissues.

Programmed cell death, or apoptosis, plays a critical role in normal spermatogenesis. Previous studies have proved that oxidative stress induced by a high glucose concentration triggers events associated with the proapoptotic protein Bax, including subsequent caspase activation and an abnormal progression to apoptotic cell death. In addition, hyperglycemia downregulates the glucose transporters GLUT1, GLUT2, and GLUT3, triggering the mitochondrial death cascade pathway. An interdependent relationship has been established between increased levels of FBG, HbA$_1c$, and overexpression of Bax protein that magnifies cell death by endothelial dysfunction in diabetes. Present findings from the densitometric analysis of testicular proapoptotic Bax-α protein expression strongly support all these relationships.

The increased fructose levels in seminal plasma seen in diabetes may be due to impaired fructose utilization in line with a low sperm count, which has also been shown in previous works. Our results showed that, in diabetes, the tuning system of the pituitary–testicular axis has been blunted and finally leads to infertility.

Previous reports have shown that diabetes mellitus cannot be totally managed by a single chemical or ingredient but by multiple chemicals or ingredients, as supported by our results of treatment with the polyherbal formulation MTEC (modified). The effect might be due to a restoration of serum insulin levels towards control values, which is also supported by our findings and is in parallel with our previous reports. Phytoingredients such as alkaloids, flavonoids, and gallic acid from the different plant parts used to constitute MTEC (modified) have high antioxidant and antibacterial capacities that help this polyherbal formulation to offer protection against diabetes and its related complications. Elevated insulin levels and glycosgen levels in hepatic tissue and skeletal muscle, as well as a restoration of the activities of the carbohydrate metabolic enzymes that are under the positive regulation of insulin. In parallel, the elevation in the activities of glucose-6-phosphatase and lactate dehydrogenase in the above tissues in diabetes again supports hypoinsulinemia as these sensors are under the negative control of insulin.

The significant diminution in reproductive organosomatic indices in the diabetic rats might be due to low levels of serum testosterone and insulin. The diabetic state reduces testosterone levels by suppressing the secretion of luteinizing hormone and follicle-stimulating hormone. Impairment of spermatogenesis in diabetic rats may be due to an impairment of function of the testicular Sertoli cells, which secrete androgen-binding protein that retains testosterone in the seminiferous tubules upon stimulation with follicle-stimulating hormone. Hypoinsulinemia in diabetes also alters the expression of aromatase enzymes in the testis, which help the synthesis of testicular estradiol, an indisputable regulator of spermatogenesis.

Low sperm count and motility in diabetes may be due to low serum insulin as insulin is one of the regulators of testicular steroidogenesis. The high level of blood glucose in diabetes produces superoxide anions and hydroxyl radicals in the presence of transition metal ions, which cause oxidative damage to cell, that results in the diminished activities of the antioxidative enzymes and the elevated levels of TBARS and CD in reproductive tissues, which results in impaired spermatogenesis. The result also may be due to low levels of serum insulin and testosterone.

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of insulin after treatment with MTEC (modified), also indicate the insulinotropic activity of this polyherbal formulation.\textsuperscript{15,16}

The mechanism of beta-cell protection from the deleterious effect of STZ by MTEC (modified) may be explained by the fact that the phytoingredients present in the plant parts may increase the production of transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) in the plasma in type 1 diabetes. This contributes to the formation of a TGF-\(\beta_1\) ring around the pancreatic islets that protects the beta-cells against infiltrating lymphocytes, providing a safe environment for the promotion of beta-cell regeneration and increased serum insulin.\textsuperscript{51} Another possible mechanism in this concern is the inhibitory effect on hepatic insulinase enzyme activity that increases peripheral utilization of glucose by increasing plasma insulin.

![Figure 4](image)

**Figure 4** Histology of the testis (H&E stain, 400×). (A) Representative sample of testicular sections in Stage VII of spermatogenesis showing a normal arrangement of different generations of germ cells in the seminiferous tubules of the control group. (B) Representative sample of testicular sections showing a diminution in number and disarrangement of different generations of germ cells at Stage VII in the diabetic group. (C) Representative sample of testicular sections in Stage VII showing recovery of the number of different generations of germ cells in the MTEC (modified)-treated group. (D) Representative sample of testicular sections in Stage VII showing recovery of the number of different generations of germ cells in the glibenclamide-treated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Testiculo-somatic index (g%)</th>
<th>Epididymis somatic index (g%)</th>
<th>Seminal vesiculo-somatic index (g%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (control group)</td>
<td>127 ± 5.5</td>
<td>131 ± 5.9</td>
<td>3.46 ± 0.17</td>
<td>0.667 ± 0.03</td>
</tr>
<tr>
<td>Group II (diabetic group)</td>
<td>124 ± 5.4</td>
<td>116 ± 5.1</td>
<td>1.04 ± 0.05*</td>
<td>0.392 ± 0.02*</td>
</tr>
<tr>
<td>Group III (MTEC-treated group)</td>
<td>123 ± 5.4</td>
<td>129 ± 5.7</td>
<td>1.82 ± 0.11**</td>
<td>0.548 ± 0.03**</td>
</tr>
<tr>
<td>Group IV (glibenclamide-treated group)</td>
<td>122 ± 5.4</td>
<td>127 ± 5.6</td>
<td>1.79 ± 0.11**</td>
<td>0.519 ± 0.02**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error, \(n = 6\). Analysis of variance followed by a multiple comparison two-tail \(t\) test.

*Significant difference (\(p < 0.05\)) compared with the control group.

**Significant difference (\(p < 0.05\)) after treatment in each group compared with the control group.

***Significant difference (\(p < 0.05\)) after treatment in each group compared with the control group.
release insulin-containing granules. MTEC formulation calcium to the transport protein calmodulin, which helps to cellular calcium to enter into the cell. Increased polarization of the cell membrane, which enables extra-inhibits ATP-sensitive potassium channels, and causes de-insulin as a response to serum glucose level. Glibenclamide reduced blood glucose levels. It increases the release of and reduces hepatic glucose production, resulting in diabetes in rats with partially preserved synthesis of insulin. 52

Table 5 Study of general and metabolic toxicity biomarkers (serum glutamate pyruvate transaminase and glutamic oxaloacetic transaminase activities) after treatment with increasing doses of the polyherbal formulation MTEC (modified) in normoglycemic Wistar rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SGPT (U/L)</th>
<th>SGOT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control group)</td>
<td>35.2 ± 1.56</td>
<td>12.9 ± 0.47</td>
</tr>
<tr>
<td>Group II (90 mg MTEC treated group)</td>
<td>36.0 ± 1.56</td>
<td>13.0 ± 0.47</td>
</tr>
<tr>
<td>Group III (100 mg MTEC treated group)</td>
<td>36.2 ± 1.56</td>
<td>13.4 ± 0.47</td>
</tr>
<tr>
<td>Group IV (300 mg MTEC treated group)</td>
<td>37.3 ± 1.57</td>
<td>13.0 ± 0.47</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard error, n = 6. Analysis of variance followed by a multiple comparison two-tail t test. Values do not differ from each other (p > 0.05) when compared with the control group.

Although glibenclamide is a common drug of choice in the treatment of type 2 diabetes, its hypoglycemic effect in STZ-induced diabetes, a model of type 1 diabetes, is also well documented. 52-54 In studies of insulin-dependent diabetes in rats with partially preserved synthesis of insulin treated with glibenclamide stimulates insulin secretion and reduces hepatic glucose production, resulting in reduced blood glucose levels. It increases the release of insulin as a response to serum glucose level. Glibenclamide binds to the surface receptors of the beta-cell membrane, inhibits ATP-sensitive potassium channels, and causes depolarization of the cell membrane, which enables extra-cellular calcium to enter into the cell. Increased intracellular calcium concentrations enhance the binding of calcium to the transport protein calmodulin, which helps to release insulin-containing granules. 55 MTEC formulation may also stimulate insulin secretion following this phenomenon.

A recovery of organo-somatic indices, correction of the sperm count and motility, and arrangement of different stages of germ cells in spermatogenesis in the seminiferous tubules toward control values were noted after treatment with our polyherbal formulation or glibenclamide. This might be due to a correction of serum insulin levels that restored the testicular androgenesis process. 30 This elevated insulin level corrected the process of spermatogenesis by enhancing the upregulation of testosterone synthesis, similar to other experiments in which insulin injections to diabetic mice gave rise to an increase in serum gonadotropin and testosterone levels. 56 It was also supported in our study by a rise in the level of serum testosterone and a lowering of testicular cholesterol level, along with a histologic study of testicular sections in Stage VII of spermatogenesis showing a normal arrangement of the different generations of germ cells in the seminiferous tubules.

Testicular oxidative stress in the diabetic state that results in spermatogenic impairment recovered toward control levels after treatment with MTEC (modified) same as glibenclamide. Both the polyherbal formulation and glibenclamide treatment reduced blood glucose level, increased antioxidant enzyme activity, 29 decreased the formation of ROS, and protected target cells from oxidative damage by scavenging the end-products of free radical generation (CD and TBARS). Treatment also inhibited expression of the proapoptotic protein Bax and increased activities of SOD, peroxidase, catalase, and GST enzymes, 58 and prevents microvascular complications that produce oxidative stress. 59 This might be explained by the phenomenon of "glycemic or metabolic memory," 60 and this glycemic memory might be responsible for the ameliorative activity of MTEC or glibenclamide; this is supported by the elevated levels of CD and TBARS in diabetic rats with poor glycemic control and a normalization of these parameters in treated diabetic rats, leading to good glycemic regulation compared to controls. 61

Alkaloids, flavonoids, and gallic acid, the phytoingredients of the different plant parts used to constitute MTEC (modified), have high antioxidant capacities, 16,49,50 and this helped this polyherbal formulation to offer protection against oxidative stress. The antipapoptotic potentiality against elevated Bax protein expression was attenuated by MTEC (modified) or glibenclamide by insulinoergic activity as an inverse relationship between insulin and Bax protein expression has been established in STZ-induced diabetic rats. 62

The decrease in level of seminal vesicular fructose after treatment with MTEC (modified) demonstrated the correction in fructose synthesis, and its utilization by sperm was reflected in an increased sperm function count and motility as it is the main biofuel for sperm.

MTEC (modified) has no toxicity shown here in the acute toxicity study, as well as in the study of serum GOT and GPT activities, these being indicators of general and metabolic toxicity. 18

Conclusions

Our formulated modified MTEC offered remedial proficiency in terms of glycemic homeostasis as well as attenuation of reproductive dysfunction in a comparatively low dose in comparison to our previously formulated MTEC. The possible mode of action of this polyherbal formulation in producing a therapeutic effect in diabetes is the enhancement of insulin secretion by a regeneration of beta-cells in the pancreatic islets; this is supported here by an elevation in serum insulin level and an upregulation of carbohydrate metabolic enzymes that are under positive regulation by insulin. This rectifies the FBG level and HbA1c level and results in the re-establishment of homeostasis of carbohydrate metabolism. In terms of the restitution of the hist架构和 androgenic profile of the testicular tissue during oxidative stress and in terms of apoptotic mechanisms, MTEC (modified) improves sperm quality parameters and rectifies diabetes-induced male infertility. Its biosafety profile has also been established.
Acknowledgment

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References


