

Research Article



Herbal Blend of Cinnamon, Ginger, and Clove Modulates Testicular Histopathology, Testosterone Levels and Sperm Quality of Diabetic Rats

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ABSTRACT

Diabetes is a global problem and number of those affected is increasing day by day. This encourages researchers to find new methods to control such disease. Herbal extracts may offer some protection against the early stage of diabetes mellitus and the development of complications. The present study investigated the effect of herbal mixture of cinnamon, ginger, and clove on blood glucose levels, histological picture of testis, expression of caspase-3 and vascular endothelial growth factor (VEGF) in streptozotocin (STZ)-induced diabetic rats. Adult male rats were randomly assigned to experimental 4 groups: control group, herbal blend (HB) group, STZ induced diabetic group, and diabetic plus herbal mixture group. HB administration to diabetic rats showed hypoglycemic effect, appeared in the decrease in blood glucose levels, improvement histological testicular picture, decreased caspase-3 and VEGF expression, restore testosterone level, increased sperm count, motility and reduce sperm abnormality as compared to diabetic rats.

Keywords: Herbal mixture, Cinnamon, Ginger, Clove, Diabetic rats.

INTRODUCTION

Diabetes is a common health problem and a serious metabolic disorder associated with many functional and structural complications. Diabetes mellitus has adverse effects on male sexual and reproductive functions in diabetic patients and animals.¹ Diabetes-related effects on testicular function have been attributed to the lack of insulin. The regulatory action of this hormone is known, and observations of a direct effect on both Leydig cells² and Sertoli cells³ have been reported. Diabetes mellitus is a group of disorders with different etiologies. It is characterized by disturbances in carbohydrate, protein and fat metabolism, caused by the complete or relative insufficiency of insulin secretion and insulin action.⁴ Approximately, 150 million people worldwide suffer from diabetes.⁵ The disease become a real problem of public health in developing countries, where its prevalence is increasing steadily. In those countries, adequate treatment is often expensive or unavailable. Alternative strategies to the current modern pharmacotherapy of diabetes mellitus are urgently needed⁶, because of the inability of existing modern therapies to control all the pathological aspects of the disorders, as well as the enormous cost and poor availability of the modern therapies for many rural populations in developing countries. Many herbal preparations alone or in combination with oral hypoglycemic agents, sometimes produced a good therapeutic response in some resistant case where modern medicines alone fail.⁷ Changing the diet helps to prevent development of diabetes and to control blood glucose concentrations. Traditional herbs and spices also can be used to control blood glucose concentrations.⁸ Herbal drugs are prescribed widely because of their

effectiveness, less side effects and relatively low cost.⁹ Therefore, investigation on such agents from traditional medicinal plants has become more important. Subash Babu¹⁰ indicated that natural herbs possessed hypoglycemic and hypolipidemic effects in STZ-induced diabetic rats.

Cinnamomum cassia has been reported to be effective in the alleviation of diabetes through its antioxidant and insulin-potentiating activities. The water-soluble polyphenolic oligomers found in cinnamon are thought to be responsible for this biological activity.¹¹ Hassan¹² demonstrated the anti-diabetic, and hypolipidemic activities of cinnamon in diabetic rats. Cinnamon belongs to the Lauraceae family, and its main components are cinnamic aldehyde, cinnamic acid, tannin and methylhydroxychalcone polymer. Cinnamon also exhibits insulin-potentiating capabilities and therefore may have beneficial effects on cellular glucose uptake. Cinnamon bark possesses significant anti-allergic, anti-ulcerogenic, antipyretic and antioxidant properties.¹³ Ginger rhizome (*Zingiber officinale* R., family: Zingiberaceae) is used medicinally and as a culinary spice. The medicinal use of ginger dates back to ancient China and India.¹⁴ Ginger and its constituents are stated to have antiemetic, antithrombotic, anti-hepatotoxic, anti-inflammatory, stimulant, cholagogue and antioxidant. Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic diabetes mellitus. Both antioxidant, and androgenic activity of ginger were reported in animal models.¹⁵ All major active ingredients of *Z. officinale*, such as zingerone, gingerdiol, zingibrene, gingerols and shogaols, have antioxidant activity. *Eugenia caryophyllus* (clove), belonging to the family Myrtaceae, has a number



of medicinal properties and its systemic as well as local use has been advocated in traditional medicine. Clove is reported to possess antibacterial¹⁶, anti-pyretic¹⁷, local anesthetic¹⁸ and antioxidant¹⁶ activities. It is widely used as an aromatic stimulant, antispasmodic and carminative spice. Clove contains eugenol, acetyleugenol, sesquiterpenes, tannins, sitosterol, stigmasterol and small quantities of esters, ketones.¹⁹

Thyroid hormones (T4 and T3) are an important factor for Leydig cells steroidogenesis, and the thyroid hormones deficiency could be correlated with testis dysfunction as a result of hypertrophy and hypoplasia of Leydig cells, which in turn leads to the decrease in testosterone levels.²⁰

Caspases are central components of the machinery responsible for apoptosis. Caspase-3 is the major effector caspase involved in apoptotic pathways.²¹ VEGF is a mitogen and survival factor for vascular endothelial cells; while also promoting vascular endothelial cell and monocyte motility. Moreover, VEGF selectively and reversibly permeabilizes the endothelium to plasma and plasma proteins without leading to injury. All of these properties are required for angiogenesis. VEGF, which contains an N-linkage glycosylation site, consists of nine isoforms that result from alternative splicing of pre-mRNA transcribed from a single gene containing eight exons.²² VEGF mRNA and protein are expressed in many tissues and organs.²³ They reported that guinea pig adrenal, heart, and kidney also express high levels of VEGF mRNA while gastric mucosa, liver, and spleen express lower levels of these transcripts. Moreover, VEGF mRNA and protein are expressed in a wide variety of human malignancies including those of breast, colorectal, non-small cell lung, and prostate carcinomas.²⁴

As a result of the side effects associated with diabetes, there is an increasing interest in the exploring the effects of herbal remedies to treat this disease. The main objectives of this study were 1) to determine the effects of STZ-induced diabetes on testicular structure and function in rats; and 2) to evaluate the role of natural herb mixture in improving histological, glucose levels, T3, T4, testosterone levels, and sperm activity after the induction of diabetes.

MATERIALS AND METHODS

Source of Chemicals

STZ was purchased from Sigma Aldrich Chemical Company, (St. Louis, MO, USA). Cinnamon, ginger, and clove were purchased from commercial sources in Arar, KSA.

Experimental Animals

Male rats *Rattus rattus* were purchased from Animal House, Faculty of Medicine, King Abdul-Aziz University (Jeddah, KSA). The rats were kept in clean and dry plastic cages, with 12-hrs light-dark cycle at 25 ± 2 °C. The standard diet and tap water were available *ad libitum*

throughout the study, without any restriction to food and drinking water. The rats were assigned into 4 groups of 7 rats each. Rats in group 1 were the control which received normal saline. Animals belonging to group 2 received herbal blend (HB) orally. Diabetes was induced in groups 3 and 4 using STZ. After three days of diabetes induction, animals with blood glucose levels greater than 300 mg/dl were considered as diabetic and selected for the study. Animals from group 4 received daily HB extract for 15 days, after the induction of diabetes.

Induction of Diabetes

STZ was freshly dissolved in 10 mM sodium citrate (pH 4.5) at a dose level of 50 mg/kg b.w.²⁵, and injected intraperitoneal, within 15 min of dissolution. After the administration of STZ, the animals were given 1% sucrose solution to prevent hypoglycemia. Blood glucose were measured 3 days later, by Bayer's Contour meter (Japan) to determine hyperglycemia. Rats with blood glucose levels greater than 300 mg/dl were considered as diabetic and selected for the study.

Preparation of Herbal Aqueous Extract

Ten grams of cinnamon, ginger and clove powder boiled in 100ml of distilled water. The solutions allowed to dry and stored in dark bottles and used daily for 15 days. Each 2 ml of solution equivalent to 200 mg of cinnamon, ginger, and clove according to Jia.¹¹, Li²⁶ and Singh²⁷ respectively.

Body Weights

Animals were individually weighed by means of a Meopta sensitive balance. Whole body weights were recorded to the nearest 1 mg to determine weekly changes.

Testis Weights

Fresh right and left testes from autopsied rats were blotted dry and subsequently weighted. Absolute testis weights were recorded to the nearest 0.1 mg by using an electric balance.

Blood Sampling and Biochemical Analyses

Blood samples were collected from the heart by sterile syringes and left to clot for separating the serum after centrifugation at 3000 rpm for 15 minutes. Serum samples were directly frozen at -10 °C till biochemical analyses. Estimation of blood glucose was carried out using enzymatic glucose kits from Human Gesellschaft für Diagnostica mbH, Germany. Serum level of thyroid hormones: total triiodothyronine (T3), total thyroxine (T4) and testosterone were BioMérieux kits, France.

Histological Examinations

Testicular tissues were excised from sacrificed animals, weighed, and fixed in 10% neutral buffer solution and were sequentially embedded in paraffin wax blocks according to the standard procedure, sectioned at 5µ thickness. They were further deparaffined with xylol, and histologically observations were performed, using H-E



technique. The slides were examined using light microscope.

Immunohistochemical Investigations

The advantage of the immunohistochemical methods is the ability to show exactly where a given protein is located. Blocks were cut into 4 mm thick sections mounted on glass +slides, and incubated at 4 °C overnight. Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed. Slides were probed with either anti-caspase-3 (ab4051) or anti-VEGF (ab46154). Sections were washed three times with PBS for 10 min each and incubated with biotin-labeled anti-mouse IgG for 1 h at room temperature. After washing, sections were stained with a streptavidin-peroxidase detection system according to the manufactured instructions. The antibodies purchased from ABCAM, Cambridge Science Park in Cambridge, England.

Sperm Activity

For determination sperm motility left epididymis was placed in pre-warmed Petri-dish containing 5ml of sodium citrate solution (2.9%) at 37 °C. The cauda was then nicked with a scalpel blade to allow sperm to emerge from the engorged cauda epididymis, and placed in 37 °C incubator for 15 min. The suspension was stirred and one drop placed on a warmed slide with a cover slip placed. At least five microscopic fields were observed at 400X magnification, using a standard light microscope. Motility was reported as an average percent motile. Morphological abnormality of sperms done by taken one drop of the previous suspension of live sperm, placed on a slide, and a smear was made by using the edge of another slide, as procedure for preparing blood smears. The slides were air dried and stained with 1% eosin at room temperature. Approximately 200 sperm from each rat were examined by light microscopy and classified as normal and abnormal. For sperm counting, right epididymis and specimens of right testis were homogenized manually in 0.5 ml of 0.9% NaCl solution. The homogenates were diluted with 1.5 ml of saline, spermatozoa were counted using Neubauer haemocytometer at 400X magnification in five squares as WBCs. Five counts/sample were averaged.²⁸

Statistical Analysis

Data were presented as Mean \pm SE and were analyzed using one-way analysis of variance (ANOVA) by the Statistical Processor System Support "SPSS" for Windows software, version 16.0 (SPSS, Chicago, IL), to compare all the treated groups. Once a significant F test was obtained, followed by post hoc-least significant difference analysis (LSD) was performed; LSD comparisons were performed to assess the significance of the difference among various treated groups, with the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Morphological Investigation and General Behavior

Rats belonging to the control group showed normal behavior throughout the experimentation period. They were active by day and calm by night and kept their white furs clean (Fig. 1a). Water consumption and urine excretion were of average amounts in this group. Diabetic animals exhibited many symptoms commonly associated with diabetes such as weakness, loss appetite, polyuria, diarrhea and their fur color turned pink (Fig. 1b). They suffered from irritability, drowsiness, disturbances of sleep, and states of depression. Many studies have clearly shown cognitive and behavioral changes in type 1 diabetic rats and humans, which are evident in elevated levels of anxiety, depression, and slowing of mental speed and flexibility.^{29,30} Diabetes-induced behavioral and cognitive changes are related to several factors. Both diabetic complications and reduced central serotonin (5-hydroxytryptamine, 5-HT) synthesis and metabolism are thought to underlie behavioral and cognitive dysfunctions in patients with T1DM.³¹ Depression and anxieties are about twofold higher in diabetic patients than in the general population. Increasing evidence points toward an association between diabetes mellitus and deficit in learning and memory.³² Chronic treatment with curcumin (60 mg/kg; p.o.) significantly attenuated cognitive deficit, cholinergic dysfunction, oxidative stress and inflammation in diabetic rats. The results emphasize the involvement of cholinergic dysfunction, oxidative stress and inflammation in the development of cognitive impairment in diabetic animals and point towards the potential of curcumin as an adjuvant therapy to conventional anti-hyperglycemic regimens for the prevention and treatment of diabetic encephalopathy.³³



Figure 1(a & b): Photomicrographs for experimental animals showing the normal white furs of control rat (a), and pink furs of STZ injected rats.

Growth Rates

Body weight values were found as 205.1, 202.9 and 164.6 g in the control, HB and STZ groups, respectively. As administration of HB to diabetic rats, an increase in body weight reported in animals ($p < 0.05$) reached 182.9 g when compared with diabetic animals as mentioned in Table 1.

Right testis weight recorded 2.02, 1.94 and 1.14 for control, HB and STZ respectively. The administration of HB to diabetic rats caused increase in the right weight testis reached 1.85 when compared to the diabetic group. The same result was reported for the left testis weight.

The present results reported decrease in body and testis weight in diabetic rats. This data were reversed by the administration of HB. This comes in accordance with those of Al-Amin.³⁴

The characteristic loss of body weight in STZ induced diabetes may be due to increased muscle wasting and due to loss of tissue proteins.³⁵ The cinnamon and row ginger extracts treated diabetic rats showed significant recovery in body weight when compared to single administration extract. This may be due to controlling muscle wasting and improvement in insulin secretion as well as glycemic control by the cinnamon.^{34,36}

Table 1: Body and Testes weights in Control and Experimental Groups.

Experimental groups	Control Group	HB Group	STZ Group	STZ+HB Group
Body weight (g)	205.1 ± 1.56	202.9 ± 1.61	164.6 ± 3.42 ^{a,b}	182.9 ± 1.54 ^c
% of change		1.07	19.74	10.82
Right testis weight (g)	2.02 ± 0.086	1.94 ± 0.084	1.14 ± 0.097 ^{a,b}	1.85 ± 0.061 ^c
% of change		3.96	43.56	8.41
Left testis weight (g)	1.95 ± 0.09	1.94 ± 0.09	1.11 ± 0.10 ^{a,b}	1.81 ± 0.073 ^c
% of change		0.51	43.07	7.17

Values are mean ± SE. Superscript letters denote the significant difference at ($P < 0.05$).

a: values are significantly different from control group.

b: values are significantly different from HB group.

c: values are significantly different from STZ group.

Table 2: Serum Levels of Glucose, T3, T4 and Testosterone in Control and Experimental Groups.

Experimental groups	Control group	HB group	STZ group	STZ+HB group
Glucose (mg/dl)	126.2 ± 3.82	125.1 ± 3.44	462.5 ± 15.80 ^{a,b}	132.7 ± 11.76 ^c
% of change		0.87	-266.48	-5.15
T3 (pg/ml)	7.3 ± 0.24	7.4 ± 0.19	1.3 ± 0.15 ^{a,b}	3.2 ± 0.21 ^c
% of change		-1.36	82.19	56.16
T4 (pmol/l)	13.3 ± 0.75	15.3 ± 0.66	4.84 ± 0.44 ^{a,b}	10.24 ± 0.44 ^c
% of change		-15.03	63.60	23.00
Testosterone (ng/ml)	2.38 ± 0.21	2.42 ± 0.21	0.27 ± 0.04 ^{a,b}	2.1 ± 0.19 ^c
% of change		-1.68	88.65	11.76

Values are mean ± SE. Superscript letters denote the significant difference at ($P < 0.05$).

a: values are significantly different from control group.

b: values are significantly different from HB group.

c: values are significantly different from STZ group.

Biochemical Levels

The blood glucose concentrations in the control and HB groups were 126.2 and 125.1 mg/dl respectively. Induction of diabetes led to an increase in the blood sugar level reached 462.5 mg/dl ($p < 0.05$) as shown in Table 2. Treatment of HB to diabetic rats led to reduction of the blood glucose level reached 132.7 mg/dl, which was statistically significant at ($p < 0.05$) as compared with diabetic group. Hyperglycaemia was reported in the present study due to STZ injection which caused pancreatic β cells destruction. This may led to induce oxidative stress which have been reported to occur via increased glycolysis; autooxidation of glucose and non-enzymatic protein glycation.³⁷ HB administration lead to

decrease glucose levels in diabetic rats. Many studies reported that ginger³⁴, cinnamon³⁸ and clove³⁹ have antihyperglycaemic effect by reducing glucose levels in blood in diabetic rats. This may be related to that herbal aqueous extract has been shown to increase glucose uptake and glycogen synthesis and to increase phosphorylation of the insulin receptor.³⁸

The data shown in Table 2 also recorded a non significant changes in T3, T4 and testosterone levels in serum of rats treated with HB if compared with control group. On the other hand, STZ administration revealed marked inhibition in T3, T4 and testosterone levels.

This inhibition was significant at ($P < 0.05$) if compared with control and HB groups. Significant increase levels in



T3 (3.2), T4 (10.24) and testosterone (2.1) were observed in STZ+HB samples when compared with STZ group 1.3, 4.84 and 0.27 respectively. The administration of STZ to male rats induces a decrease in T3, T4 and testicular testosterone production. This decrease may be the result of both a decrease in the total number of Leydig cells causing strong decrease in the expression of testosterone and come in agreement with Hurtado de Catalfo.² Moreover, this alteration is responsible for the diabetes-related effects on libido.⁴⁰ After STZ treatment, a significant increase in degenerated germ cells at various stages of development is observed.⁴¹ Ward.⁴² indicated that STZ-induced diabetes causes a marked decrease, not only in serum insulin but also in serum LH levels, which would explain the impairment of Leydig cell function. Sperm production is an FSH-regulated process that requires normal Sertoli cell function. The decrease in T3, T4 and testosterone hormone levels in STZ injected rats, may also be related to the decreased number in Sertoli cells and the damaged Leydig cells that appeared in the testicular tissue sections. In the rat, Sertoli cells proliferate only during the fetal and early neonatal periods before assuming a terminally differentiated state. The ultimate number of Sertoli cells in the adult testis is determined by both the rate and the duration of the proliferative phase.⁴³ It has been demonstrated that each Sertoli cell is capable of supporting a limited number of germ cells through to maturity; hence Sertoli cell number determines the maximum spermatogenic potential of the testis. The hormonal factors controlling the rate and duration of Sertoli cell proliferation are therefore critical determinants of fertility. Previous studies have demonstrated that FSH is a mitogenic factor during the Sertoli cell proliferative phase, while thyroid hormones (T3 and T4) influence the duration of proliferation.⁴³ Kim⁴⁴, reported that the thyroid hormone deficiency caused failure Leydig cells differentiation, retard Sertoli cell maturation, and decrease T3, T4, and testosterone levels. The deficiency of thyroid hormones could originate from decreased iodide pump activity resulting in the inhibition of tyrosine iodination⁴⁵ or may be due to the decrease in the synthesis of thyroxine-binding globulin, the major serum thyroid hormone-binding protein as reported by Concannon.⁴⁶ Many studies regarding point out that hypothyroidism may arrest differentiation of Leydig cell in adult testis and more importantly revealed that Leydig cells underwent atrophic changes in size and organelle content⁷⁴, thus enabling them to go into a malfunctioning status.

Histological examinations

It was observed that the testis tissue in the control group was covered with an albuginous capsule. Septa emanate from this capsule to subdivide the testis into about 250 incomplete lobules. Each lobule contains one to four seminiferous tubules embedded in a connective tissue stroma. These tubules are enclosed by a thick basal lamina and surrounded by 3-4 layers of smooth muscle cells. The insides of the tubules are lined with

seminiferous epithelium, which consists of two general types of cells: spermatogenic cells and Sertoli cells (Fig. 2a). The histological structure in the HB treated revealed no histopathological changes as compared with control group (Fig. 2b). The seminiferous tubule structure in the diabetic rats was found to be disrupted, and there was a considerable decrease in the spermatogenic cell series. The histopathological damage of the testicular tissue included deformed seminiferous tubules (Fig. 2c). Loss and degeneration of all types of the spermatogenic cells within the seminiferous tubules without sperms at its lumen (Fig. 2d), decrease the numbers of all types of the spermatogenic cells and pyknotic Leydig cells (Fig. 2e). The number of spermatogenic cells in the HB-treated diabetic rats was observed to be increased compared to the diabetes group, and there was mild improvement in the seminiferous tubule structure as compared with the former group, this may due to the short term of treatment. The improvement appeared by the restoration of the spermatogenic cells, which are found in an arranged series and the presence of the sperms (Fig. 2f). Histopathological damages were reported in testicular tissue due to the injection of STZ, which reversed by the administration of HB. Prakasam⁵¹ have reported that STZ generated lipid peroxidation and DNA breaks in pancreatic islet cells, induced diabetes mellitus. Also it has been observed that insulin secretion is closely associated with lipoxygenase derived peroxides.⁵² Increase in lipid peroxidation, is an indirect evidence of intensified free radical production.⁵³ Oxidative stress is caused by generation of free radicals such as ROS. In physiological condition, the body synthesizes both free radicals and antioxidants.⁵⁴ Oxidative stress sets in when an imbalance occurs between these free radicals and antioxidants in favor of the free radicals. Additionally, an experimental study indicated that diabetes mellitus produces reproductive dysfunction.⁵¹ Apoptosis, known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations. Apoptosis control is critical for normal spermatogenesis in the adult testes. The testis is sensitive to environmental exposure-induced cellular damage. Apoptosis of germ cells may occur during non-physiological stresses such as diabetes.⁵⁵ Chandrashekar & Muralidhara⁵⁶ showed the occurrence of oxidative impairments and their progression in the testis of diabetic adult rats, which caused severe testicular atrophy. STZ-injection leading testis to become more vulnerable to oxidative stress, which may play a significant role in the development of testicular degeneration, leading to impaired fertility in adulthood. On the other hand, other researches showed that ginger oil has dominative protective effect on DNA damage induced by H₂O₂ and might act as a scavenger of oxygen radical and might be used as an antioxidant.⁵⁷ Antioxidants protect DNA and other important molecules from oxidation and damage, and can improve sperm quality and consequently increase fertility rate in men.⁵⁸ Therefore, the role of nutritional and biochemical factors



in reproduction and sub-fertility treatment is very important. Again, Sağlam⁵⁸ and Nassiri¹⁴ indicated that administration of ginger and cinnamon to STZ-induced rats may provide benefit against diabetic conditions, by increasing the activities superoxide dismutase, glutathione peroxidase (GPx), and catalase (CAT), MDA and TAC.

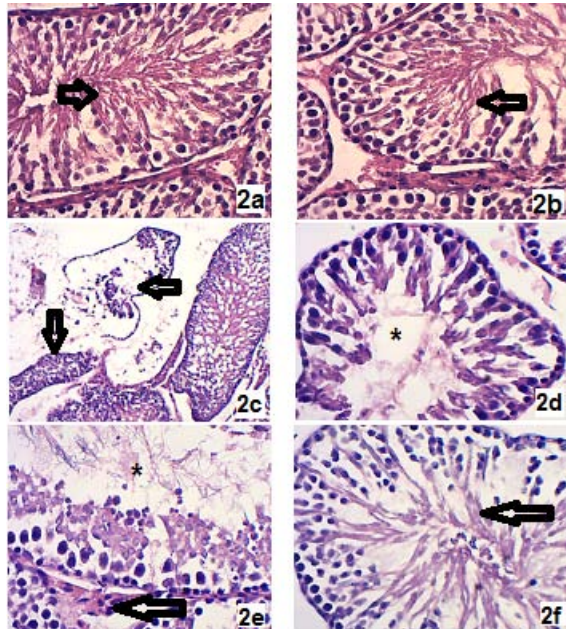


Figure 2(a-b): Photomicrographs of testis from control and HB treated rats showing normal testicular architecture with different series of spermatogenic cells (a) with sperms (arrows) (b) (H-E, X400).

Figure 2(c-e): Photomicrographs of testis from STZ injected rats showing deformed seminiferous tubules (arrows) (c; H-E, X200). Loss and degeneration of all types of the spermatogenic cells within the seminiferous tubules without sperms at its lumen (*) (d; H-E, X400). Decrease the numbers of all types of the spermatogenic cells and pyknotic Leydig cells (arrow) (e; H-E, X 400).

Figure 2f: Photomicrograph of testis from rats treated with STZ+HB showing restoration of the spermatogenic cells, which are found in an arranged series and the presence of the sperms (arrow) (g, H-E, X 400).

Immunohistochemical Study

Caspase-3

The present study preferred to confirm the apoptosis with active caspase-3 immunostaining. In the present study, the testicular cells inclined to undergo apoptosis were distinctively marked in the sections stained with active caspase-3 in STZ group (Fig. 3c). Active caspase-3 positive cells were lower in the STZ+HB group (Fig. 3d) when compared to STZ group, and it was seen that the treatment had a positive effect. This is the first study demonstrates the anti-apoptotic effects of HB against testicular injury in diabetic rats. Caspase-3 protein is localized to germ cells (Fig. 3a & b), and that after reduction of intratesticular testosterone, intense caspase-

3 immunoreactivity translocates from the cytoplasm to become concentrated in the nuclei of spermatocytes. The present work demonstrated that the administration of HB caused a significant decrease in active caspase-3-positive cells on testis tissue.

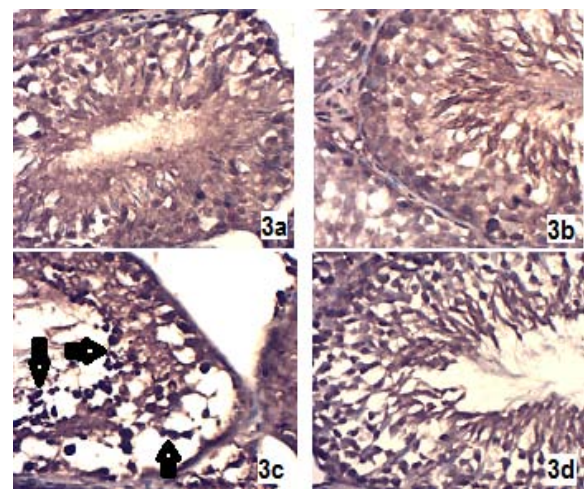


Figure 3(a-d): Photomicrograph of testes showing normal distribution of caspase-3 from control rat (a) and from HB treated rat (b) in the cytoplasm of the germ cells. Intense immunoreactivity was seen primarily in the nuclei of spermatocytes of caspase-3 in all seminiferous tubule cell series (arrows) from STZ injected rat (c). Decrease in caspase-3 positive cells as compared with STZ group (arrow) from rat treated with STZ+HB (d). (Immunohistochemical stain, X400).

VEGF

The immunohistochemical staining of VEGF in testis sections showing increase in its expression in testis from diabetic testis (Fig. 4c) as compared with the control (Fig. 4a) and HB (Fig. 4b). The expression decrease in STZ+HB treated rats when compared with STZ injected rats (Fig. 4d).

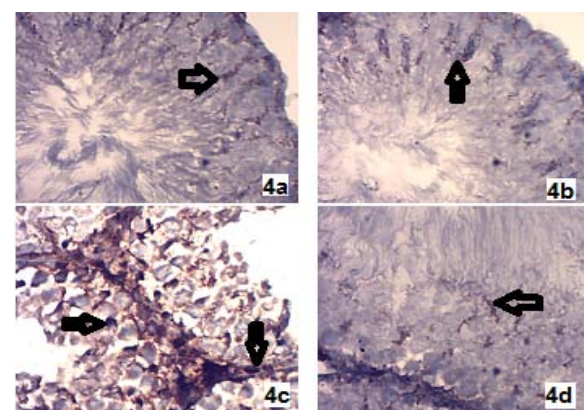


Figure 4 (a-d): Photomicrographs of testis showing normal distribution of VEGF in the testicular tissue (arrows) from control rat (a) and HB treated rat (b). Increase expression of VEGF in the apoptotic spermatocytes and Leydig cells (arrows) from STZ injected rats (c). Decrease in VEGF expression when compared with STZ injected rats (arrow) from rat treated with STZ+HB (d). (Immunohistochemical stain, X400).

Increased apoptosis in rat testicular tissue was reported in this study due to STZ injection, as appeared by the increased expression of caspase-3. This increase was declined as a result of HB administration. This come in accordance with Cai⁵⁵ who showed that the increased apoptosis in the seminiferous tubule of STZ-induced diabetic mice and rats, as a result of the oxidative stress. Caspase-3 protein is localized to germ cells, and that after reduction of intratesticular testosterone, intense caspase-3 immunoreactivity translocates from the cytoplasm to become concentrated in the nuclei of spermatocytes. This observation suggests that, as in Leydig cells, nuclear translocation of activated caspase-3 may be required for caspase-3 to function in germ cell apoptosis.⁶⁰ These results suggested that germ cell apoptosis resulting from reduced intratesticular testosterone concentration is caspase-3 activation dependent, and further, that the translocation of active caspase-3 to the nucleus may be a prerequisite for DNA degradation and subsequent germ cell apoptosis.⁶¹ The increase in VEGF expression in diabetic testicular tissue was also reported immunohistochemically by Hou & Song.⁶² The authors reported that the administration of *Thallus Laminariae* to diabetic rats repaired VEGF expression.

Sperm Motility, Abnormality and Count

STZ-induced diabetic by 50 mg/kg significantly decreased sperm motility and count, and increased abnormality as compared with those observed in the control and HB groups. This values were significant at ($p < 0.05$). The corresponding values in STZ+HB treated group were significantly improved as shown in Table 3 as compared with STZ group. The abnormal sperm morphology (158.88%) was represented as sperms with curved, double, lobed, flattened and pear-shaped heads (Fig. 5 c-g).

Erukainure⁴⁸ revealed that the testis contains an elaborate array of antioxidant enzymes and free radical scavengers to protect against oxidative stress. This is of great importance as peroxidative damage is currently regarded as the major cause of impaired testicular functions.⁴⁹ Hyperglycaemia have been reported to be associated with testicular failure leading to sexual dysfunction, impotence and infertility.^{34,37} The lipids in

sperm are the main substrates for peroxidation, which is induced by reactive oxygen species (ROS) generated by hyperglycaemia. This will ultimately lead to infertility in most men, characterized by low sperm count and motility. Increasing evidence suggests that diabetes has an adverse effect on male reproduction function and oxidative stress may be involved.⁵⁰ However, administration of ginger significantly increased sperm motility and viability as compared with the diabetic group. These results are supported by the finding Nassiri¹⁴, who reported that this increase in sperm motility could be due to the protective effect of ginger. Beside, this productive effect is reflected by the decrease of malondialdehyde (MDA) and increase in total antioxidants capacity (TAC) levels. Insufficient sperm motility is a common cause of infertility, thus the improved motility due to blend of pawpaw, guava, and grape fruit administration signifies improvement of the sperm quality and improved fertility. The observed improvement of the sperm count represents its potency in the management of the diabetic-induced spermatocic and impotency in males.⁴⁸ This could be attributed to the ability of the blend of pawpaw, guava, and grape fruit to improve the testicular antioxidant activities as evidenced by the enhanced antioxidant markers.

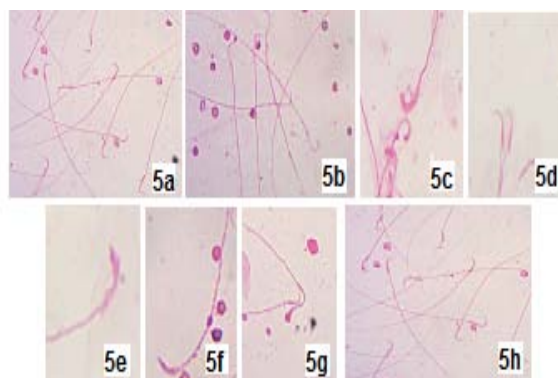


Figure 5(a-h): Photomicrographs of sperm smear showing normal sperms with hooked heads from control rat (a) HB treated rat, (b) Curved heads without hook, (c) double head sperm, (d) lobed heads, (e) flattened head, (f) pyriform or pear-shaped, (g) from STZ injected rats and normal hook-shaped heads, (h) from rat treated with STZ+HB (H-E).

Table 3: Sperm Motility, Abnormality, and counting of the Experimental Groups.

Experimental groups	Control group	HB group	STZ group	STZ+HB
Sperm Motility (%)	60.42 ± 3.30	65.71 ± 2.38	22.57 ± 1.70 ^{a,b}	50.71 ± 2.56 ^c
% of change		-8.75	62.64	16.07
Sperm Abnormality (%)	5.57 ± 0.36	5.28 ± 0.35	14.42 ± 1.21 ^{a,b}	8.0 ± 0.53 ^c
% of change		5.20	-158.88	-43.62
Sperm Counting (x 10 ⁶ /ml)	137.57 ± 4.09	136.00 ± 4.53	46.42 ± 2.28 ^{a,b}	131.43 ± 4.66 ^c
% of change		1.14	66.25	4.46

Values are mean ± SE. Superscript letters denote the significant difference at ($P < 0.05$).

a: values are significantly different from control group.

b: values are significantly different from HB group.

c: values are significantly different from STZ group.



CONCLUSION

The above mentioned findings suggested that the increased use of herbal medicine can decrease the side effects of diabetes mellitus on male hormone, sperm parameters and histopathological damage. To our knowledge, this is the first report indicating that herbal blend of ginger, cinnamon and clove improved diabetes induced testicular dysfunction in diabetic rats. HB can contribute to a balanced oxidant-antioxidant status and provide a useful therapeutic option to reduce the associated testes injury in patients with diabetes mellitus. The use of herbal mixture with treatment prescribed by a doctor pays the results in lowering blood sugar levels to normal levels.

REFERENCES

- Abdul Nabi S, Kasetti RB, Sirasanagandla S, Tilak TK, Kumar MVJ, Rao CA, Antidiabetic and antihyperlipidemic activity of *Piper longum* root aqueous extract in STZ induced diabetic rats, *BMC Complementary and Alternative Medicine*, 2013, 2013, 13-37.
- Hurtado deCatalfo G, Nelva I, DeGo'mez Dumm T, Lipid dismetabolism in Leydig and Sertoli cells isolated from streptozotocin-diabetic rats, *International Journal of Biochemistry and Cell Biology*, 30, 1998, 1001-1010.
- Mita M, Borland K, Price JM, Hall PF, The influence of insulin and insulin-like growth factor-I on hexose transport by Sertoli cells, *Endocrinology*, 116, 1985, 987-992.
- Ghazanfar K, Ganai BA, Akbar S, Mubashir K, Ahmad Dar S, Younis Dar M, Tantry MA, Antidiabetic Activity of *Artemisia amygdalina* Decne in Streptozotocin Induced Diabetic Rats, *BioMed Research International*, 2014, 2014, 1-10.
- WHO, Diabetes mellitus, Fact sheet number 138 and 236 Geneva, 1999.
- WHO, launches the first strategy on traditional medicine press release WHO/38, 2002.
- Anturlikar SD, Gopumadhavan S, Effect of D-400 a formulation on blood sugar normal and alloxan-induced diabetic rats, *Indian Journal of physiology and pharmacology*, 39, 1995, 95-100.
- Hlebowicz J, Darwiche G, Björgell O, Almér LO, Effect of cinnamon on postprandial blood glucose, gastric emptying, and satiety in healthy subjects, *American Journal of Clinical Nutrition*, 85(6), 2007, 1552-1556.
- Suba V, Muragesan T, Bhaskara RR, Ghosh L, Pat M, Mandal SC, Saha BP, Antidiabetic potential of *Barleria lupulina* extract in rats, *Fitoterapia*, 75, 2004, 1- 4.
- Subash-Babu P, Prabuseenivasan S, Ignacimuthu S, Cinnamaldehyde - a potential antidiabetic agent, *Phytomedicine*, 14(1), 2006, 15-22.
- Jia Q, Liu X, Wu X, Wang R, Hu X, Li Y, Huang C, Hypoglycemic activity of a polyphenolic oligomer-rich extract of *Cinnamomum parthenoxylon* bark in normal and streptozotocin-induced diabetic rats, *Phytomedicine*, 16(8), 2009, 744-750.
- Hassan SA, Barthwal R, Nair MS, Haque SS, Aqueous Bark Extract of *Cinnamomum Zeylanicum*: A Potential Therapeutic Agent for Streptozotocin-Induced Type 1 Diabetes Mellitus (T1DM) Rats, *Tropical Journal of Pharmaceutical Research*, 11(3), 2012, 429-435.
- Solomon TPJ, Blannin AK, Effect of short-term cinnamon ingestion on in vivo glucose tolerance, *Diabetes, Obesity and Metabolism*, 9(6), 2007, 895-901.
- Nassiri M, Khaki A, Ahmadi-Ashtiani HR, Rezazadeh Sh, Rastgar H, Gharachurlu SH, Effects of Ginger on Spermatogenesis in Streptozotocin-induced Diabetic Rat, *Journal of Medicinal Plants*, 8(31), 2009, 118-124.
- Palmeira CM, Santos DL, Seica R, Moreno AJ, Santos MS, Enhanced mitochondrial testicular antioxidant capacity in Goto-Kakizaki diabetic rats: role of coenzyme Q, *American Journal of Physiology and Cell Physiology*, 281, 2001, C1023-C1028.
- Cai L, Wu CD, Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens, *Journal of Natural Products*, 59, 1996, 987-990.
- Feng J, Lipton JM, Eugenol: Antipyretic activity in rabbits, *Neuropharmacology*, 26, 1987, 1775-1778.
- Ghelardini C, Galeotti N, Di CesareMannelli L, Mazzanti G, Bartolini A, Local anaesthetic activity of beta-caryophyllene, *Farmaco*, 56, 2001, 387-389.
- Evans WC, Trease and Evans pharmacognosy, 14th ed. London: Saunders, 2001.
- Ariyarante HBS, Mills N, Mason JI, Mendis-Handagama SML, Effect of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment, *Biology of Reproduction*, 63, 2000, 1115-1123.
- Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH, Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis, *BMC Cell Biology*, 14, 2013, 32.
- Guttman-Raviv N, Kessler O, Shraga-Heled N, Lange T, Herzog Y, Neufeld G, The neuropilins and their role in tumorigenesis and tumor progression, *Cancer Letters*, 231, 2006, 1-11.
- Maharaj AS, Saint-Geniez M, Maldonado AE, D'Amore PA, Vascular endothelial growth factor localization in the adult, *American Journal of Pathology*, 168, 2006, 639-648.
- Jr RR, Vascular endothelial growth factor (VEGF) signaling in tumor progression, *Critical Reviews in Oncology/Hematology*, 62, 2007, 179-213.
- Prasad SK, Kulshreshtha A, Qureshi, TN, Antidiabetic Activity of Some Herbal Plants in Streptozotocin Induced Diabetic Albino Rats, *Pakistan Journal of Nutrition*, 8(5), 2009, 551-557.
- Li Y, Tran VH, Duke CC, Roufogalis BD, Preventive and Protective Properties of *Zingiber officinale* (Ginger) in Diabetes Mellitus, Diabetic Complications, and Associated Lipid and Other Metabolic Disorders, *Evidence-Based Complementary and Alternative Medicine*, 2012, 2012, 1-11.
- Singh AK, Dhamanigi SS, Asad M, Anti-stress activity of hydro-alcoholic extract of *Eugenia caryophyllus* buds (clove), *Indian Journal of Pharmacology*, 41(1), 2009, 28-31.
- Ibrahim AAE, Correlation between fennel- or anise-oil administration and damage to the testis of adult rats, *Egyptian Journal of Biology*, 10, 2008, 62-76.
- Alvarez EO, Beauquis J, Revsin Y, Banzan AM, Roig P, De Nicola AF, Saravia F, Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes, *Behavior and Brain Research*, 198(1), 2009, 224-230.
- Rajashree R, Kholkute SD, Goudar SS, Effects of Duration of Diabetes on Behavioral and Cognitive Parameters in Streptozotocin-Induced Juvenile Diabetic Rats, *Malaysian Journal of Medical Science*, 18(4), 2011, 26-31.
- Thorre K, Chaouloff F, Sarre S, Meeusen R, Ebinger G, Michotte Y, Differential effects of restraint stress on hippocampal 5-HT metabolism and extracellular levels of 5-HT in streptozotocin-diabetic rats, *Brain Research*, 772(1-2), 1997, 209-216.
- Jackson-Guilford J, Leander JD, Nisenbaum LK, The effect of streptozotocin induced diabetes on cell proliferation in the rat dentate gyrus, *Neuroscience Letters*, 293, 2000, 91-94.
- Kuhad A, Chopra K, Curcumin attenuates diabetic encephalopathy in rats: behavioral and biochemical evidences, *European Journal of Pharmacology*, 576(1-3), 2007, 34-42.
- Al-Amin ZM, Thomson M, Al-Qattan KK, Peltonen-Shalaby R, Ali M, Anti-diabetic and hypolipidaemic properties of ginger (*Zingiber officinale*) in streptozotocin-induced diabetic rats, *British Journal of Nutrition*, 96(4), 2006, 660-666.



35. Shirwaikar A, Rajendran K, Kumar DC, Oral antidiabetic activity of *Annona squamosa* leaf alcohol extract in NIDDM rats, *Pharmaceutical Biology*, 42, 2004, 30-35.
36. Rekha N, Balaji R, Deecaraman M, Antihyperglycemic and antihyperlipidemic effects of extracts of the pulp of *Syzygium cumini* and bark of *Cinnamomum zeylanicum* in streptozotocin-induced diabetic Rats. *Journal of Applied Bioscience*, 28, 2010, 1718-1730.
37. Ahmed RG, The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defense system, *Journal of Islamic Academic of Sciences*, 15, 2005, 31-42.
38. Khan A, Safdar M, Khan MMA, Khattak KN, Anderson RA, Cinnamon Improves Glucose and Lipids of People With Type 2 Diabetes, *Diabetes Care*, 26, 2003, 3215-3218.
39. Sadiq S, Rasheed Z, Sadiq S, Sadiq R, Antihyperglycaemic And Antihyperlipidemic Effects Of Ethanolic Extract Of *Syzygium Aromaticum* (Clove) In Streptozotocin Induced Diabetic Rats. *British Pharmacological Society, BPS Winter Meeting, 2012, London, 18-20 December 2012.*
40. Hassan AA, Hassouna MM, Taketo T, Gagnon C, Elhilali MM, The effect of diabetes on sexual behavior and reproductive tract function in male rats, *Journal of Urology*, 149, 1993, 148-154.
41. Sanguinetti RE, Ogawa K, Kurohmaru M, Hayashi I. Ultrastructural changes in mouse Leydig cells after streptozotocin administration, *Experimental Animal*, 44, 1995, 71–73.
42. Ward DN, Bousfield GR, Moore KH, Gonadotropins. In: Cupps PT, ed. *Reproduction in Domestic Animals*. San Diego, Calif: Academic Press, 1991, 25-67.
43. Buzzard JJ, Morrison JM, O'Bryan MK, Song Q, Wreford NG, Developmental expression of thyroid hormone receptors in the rat testis, *Biology of Reproduction*, 62, 2000, 664-669.
44. Kim I, Ariyarante HBS, Mendis-Handagama SMLC, Changes in the testis interstitium of brown Norway rats with aging and effects of luteinizing and thyroid hormones on the aged testes in enhancing the steroidogenic potential, *Biology of Reproduction*, 66, 2002, 1359-1366.
45. Virion A, Dema D, Pommier J, Opposite effects of thiocyanate on tyrosine iodination and thyroid hormone synthesis. *European Journal of Biochemistry*, 122, 1980, 1-7.
46. Concannon PW, Castracane VD, Rawson RE, Tennant BC, Circannual changes in free thyroxine, prolactin, testes and relative food intake in woodchucks, *Mamota monax*, *American Journal of Physiology*, 227(5), 1999, 1401-1409.
47. Mendis-Handagama SMLC, Ariyarante HBS, Teunissen van Manen KR, Haupt RL, Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism, *Biology of Reproduction*, 59, 1998, 351-357.
48. Erukainure OL, Okafor OY, Obode OC, Ajayi A, Oluwole OB, Oke OV, Osibanjo A, Ozumba A, Elemo GN, Blend of *Roselle Calyx* and Selected Fruit Modulates Testicular Redox Status and Sperm Quality of Diabetic Rats, *Journal of Diabetes and Metabolism*, 3(8), 2012, 214-219.
49. Baker HW, Reproductive effects of nontesticular illness, *Endocrinology and Metabolism Clinics of North America*, 27, 1998, 831-850.
50. Hakim P, Sani HA, Noor MM, Effects of *Gynura procumbens* extract and glibenclamide on sperm quality and specific activity of testicular lactate dehydrogenase in streptozotocin-induced diabetic rats, *Malaysian Journal of Biochemistry and Molecular Biology*, 16, 2008, 10-14.
51. Prakasam A, Sethupathy S, Pugalendi KV, Erythrocyte redox status in streptozotocin diabetic rats. Effect of *Casearia esculenta* root extract, *Pharmazie*, 58, 2003, 920-924.
52. Walsh MF, Pek SB, Possible role of endogenous arachidonic acid metabolites in stimulated release of insulin and glucagons from the isolated, perfused rat pancreas. *Diabetes*, 33(10), 1984, 929-936.
53. Maritim AC, Sanders RA, Watkins JB, Diabetes, Oxidative Stress and antioxidants, *Journal of Biochemical and Molecular Toxicology*, 17(1), 2003, 24-38.
54. Wrihten SA, Piroli GG, Grillo CA, Reagan LP, A look inside the diabetic brain: Contributors to diabetes-induced brain aging, *Biochimica et Biophysica Acta*, 1792, 2009, 444-453.
55. Cai L, Chen S, Evans T, Deng DX, Mukherjee K, Chakrabarti S, Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism, *Urology Research*, 28, 2000, 342-347.
56. Chandrashekar KN, Muralidhara K, Evidence of oxidative stress and mitochondrial dysfunctions in the testis of prepubertal diabetic rats. *Diabetes-induced testicular oxidative stress*, *International Journal of Impotence Research*, 21, 2009, 198-206.
57. Peluso MR, Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver, *Experimental Biology and Medicine (Maywood)*, 231(8), 2006, 1287-1299.
58. Shrilatha B, Muralidhara. Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: its progression and genotoxic consequences, *Reproductive Toxicology*, 23(4), 2007, 578-587.
59. Sağlam Ö, Değirmenci I, Üstüner MC, Güneş HV, The protective effects of cinnamon and sugar tea extract on diabetic rats with interrelationships between oxidative stress and DNA damage. *African Journal of Pharmacy and Pharmacology*, 6(43), 2012, 3012-3017.
60. Kim J-M, Luo L, Zirkin BR, Caspase-3 activation is required for Leydig cell apoptosis induced by ethane dimethanesulfonate, *Endocrinology*, 141, 2000, 1846-1853.
61. Kim J-M, Ghosh SR, Well ACP, Zirkin BR, Caspase-3 and Caspase-Activated Deoxyribonuclease Are Associated with Testicular Germ Cell Apoptosis Resulting from Reduced Intratesticular Testosterone, *Endocrinology*, 142(9), 2001, 3809-3816.
62. Hou Q, Song W, Protective Effect of *Thallus Laminariae* Oligosaccharide on Testis in Rats with Type 2 Diabetes Mellitus, *Traditional Chinese Drug Research and Clinical Pharmacology*, 2011, 2011, 02.

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