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PROTECTIVE EFFECT OF HESPERDIN ON TYPE I DIABETES MILLITUS INDUCED INFERTILITY IN MALE RATS

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Running Title:

Effect of hesperidin on diabetes induced infertility

ABSTRACT:

Objective:

Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important pathogenic role in chronic Type I diabetes mellitus and known to cause infertility in diabetic patients. This study examines the free radical scavenging capacity of flavonoids, hesperidin.

Materials and Methods:

Male Wistar rats (n=6) were allocated in five groups, treatment group received Hesperidin (HES) 25 and 50 mg/kg (p.o.), standard treatment group received insulin 3 IU/kg (s.c.) and diabetic group received streptozotocin (STZ) 40mg/kg (i.p.),while the normal control group did not receive any drug and were left free with food and water. Animals were kept in standard condition. On the 40^{th} day after inducing diabetes the testicular tissue of rats in whole groups was isolated and the semen samples were collected and analyzed.

Results and conclusion:

Sperm count, motility, viability and histopathological studies showed significant restoration in biochemical parameters, and in antioxidant estimations the SOD and CAT levels were significantly on the rise and LPO levels were significantly low when compared to control and treatment groups. Therefore in the study 25 mg/kg (p.o.) Hesperidin have significantly showed protective effect on sperm count, motility, viability, histopathology by reducing Reactive Oxygen Species (ROS) in

testicular tissue, so it is proved that using it can be effective for sperm parameters in healthy diabetic rat.

Keywords: Diabetes mellitus, Hesperidin, Oxidative stress, streptozotocin, sperm parameters

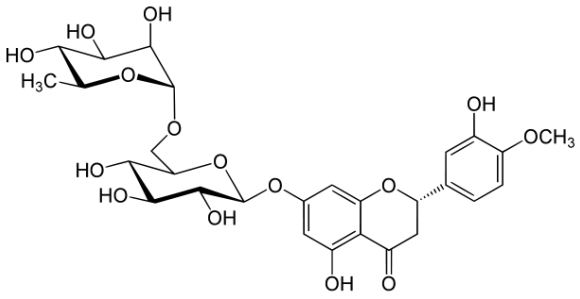
INTRODUCTION:

Diabetes Mellitus (DM) is a chronic disorder which is caused by the failure of insulin production from the Islets of langerhans in Pancreas. It is the most common endocrine disease that leads to metabolic abnormalities involving regulation of carbohydrate metabolism. These abnormalities develop pathologies including vasculopathy, neuropathy, ophthalmopathy, infertility, nephropathy and cardio myopathy (1). Hyperglycemia occurs when the human body tissue cannot store glycogen and utilise glucose. Euglycemic levels can be maintained using oral hypoglycaemic drugs, administration of insulin, treatment of obesity, controlled food intake and regular exercise and yoga (2).

Diabetes Mellitus has been associated with sexual dysfunction, both in men and women. Male infertility is one of the major health problems in life and approximately 90% of diabetic patients suffer from sexual dysfunction that includes reduced libido, ejaculation and impotence (3,4,5). Elevated Reactive Oxygen Species (ROS) and alterations in antioxidant defence mechanism play an important pathogenic role in chronic Type I diabetes mellitus. Several observational studies have reported that protection of sperm DNA damage by Reactive Oxygen Species (ROS) by consuming the antioxidants and vitamins like A, B, C & E in the diet can increase the blood flow to the testes and also increase blood testes barrier stability from altering by these free radicals (6,7).

Increased production of free radicals or ROS formation may induce oxidized Low Density Lipoprotein (Ox-LDL), which is pathogenic step in the sequence of events leading to atherosclosis. Sustained hyperglycemia and increased oxidative stress are the major pathogenic players in the development of secondary complications in diabetes.

Bioflavonoid compound *Quercetin* was proved beneficial in diabetes mellitus and also useful in infertility (8). Hesperidin (HPN, 5,7,3’-trihydroxy-4’-methoxyflavanone7-rhamnoglucoside) belongs to the class of flavonoids called flavanone is an abundant and inexpensive by-product of Citrus cultivation (9). It exhibits biological and pharmacological properties, such as antiinflammatory, anticarcinogenic, inhibit bone loss, lipid-lowering, hypoglycaemic and antioxidant activities (10,11). Un Ju Jung and his colleagues have studied hypoglycaemic effects of Hesperidin and Naringin and found that the efficacy is partly mediated by hepatic glucose-regulating enzymes in mice (12). Several studies of researchers have obsereved antioxidant activity and free radical scavenging properties of hesperidin using a variety of assay systems (13, 14, 15,16).



Accordingly, the present study is to evaluate the effect of hesperidin on male infertility in streptozotocin induced diabetic rats.

MATERIALS AND METHODS:

Chemicals:

Streptozotocin was obtained from (Himedia laboratories Pvt., Ltd., Mumbai); hesperidin was obtained from (Spectrochem Pvt., Ltd., Mumbai); Insulin was obtained from (Torrent pharmaceuticals, Mehsana); Glucometer and glucometer strips were obtained from (Aspen diagnostic (P) LTD, New Delhi) from purest grade available.

Animals:

All experiments were conducted using male wistar albino rats (150-200 g and 6-8 weeks age). All animals were procured from Sainath Agencies, Hyderabad. The animals were maintained with free access to food and water and kept at 25 ± 2°C under a controlled 12 h light/dark cycle. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. The research protocols were approved by the Institutional Animal Ethical Committee (IAEC).

Treatment groups and protocol:

The animals were divided into five groups of six (n=6) animals each. **Group I (NC)** considered as Normal control, were not administered any drug, and **Group II(DC)** served **as** Diabetic control received streptozotocin (40 mg/kg, i.p.), **Group III(DI)** served **as** Diabetic control received streptozotocin (40 mg/kg, i.p.)and a standard drug insulin (3 IU/kg, s.c.), **Group IV(DIH 25)** served **as** Diabetic control received streptozotocin (40 mg/kg, i.p.) and a standard drug insulin (3 IU/kg, s.c.) and test Hesperidin (25mg/kg, p.o.)**, Group V (DIH 50):** served **as** Diabetic control received streptozotocin (40 mg/kg, i.p.) and a standard drug insulin (3 IU/kg, s.c.) and a test drug Hesperidin (50mg/kg, p.o.).

Experimental induction of diabetes:

Rats were kept on fast overnight and injected intraperitoneally with STZ, (40 mg / kg b.wt.), freshly prepared in 0.1 M sodium citrate buffer, pH 4.5 (17). During the first 24 hours of diabetes induction, STZ-treated animals were allowed to drink 5% glucose solution to overcome drug-induced hypoglycaemia (18). Treated and control animals were allowed free access to water and standard chow diet. Seventy two hours after STZ administration, diabetes was confirmed by the presence of hyperglycemia and glucosuria. This was found respectively by means of glucometer and glucose strips. STZ-treated animals showed blood glucose more than 300 mg / dL.

Treatment schedule:

Hesperidin was suspended in 0.5% sodium carboxy methyl cellulose and administered at daily oral dose of 25 and 50 mg/kg b wt for a period of 6 weeks. In addition to these diabetic groups, two groups of normal control rats were kept without treatment till the end of the experimental period. Insulin was administered subcutaneously for 40 days alternately for three (3) days each.

Assessment of sperm parameters:

Sperm motility, viability and counts:

The caudal epididymis was dissected out; an incision (about 1 mm) was made in the caudal epididymis. Sperm fluid was then squeezed onto the microscope slide. Epididymal sperm motility was assessed by calculating motile spermatozoa per unit area and was expressed as percent motility. Epididymal sperm counts were made using the hemocytometer and were expressed as million/ml of suspension. The sperm viability was also estimated using Eosin/Nigrosin stains (19).

Histological analysis:

Testes of the treated rats were taken and fixed in 10% neutral formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. Specimens were cleared in xylene, embedded in paraffin, sectioned at 4-6 microns thickness and

stained with Hematoxylen and Eosin (H & E) dyes, and these were observed microscopically at X 100 (20).

Biochemical Estimation of Markers of Oxidative Stress:

Prior to the antioxidant activity estimations, the animals were sacrificed by decapitation. Testis were removed and rinsed with ice-cold isotonic saline. Testes were then homogenized with ice-cold 0.1 mmol/l phosphate buffer (pH 7.4). The homogenates (10% w/v) were then centrifuged at 10,000 rpm for 15 min and the supernatant so formed was used for the biochemical estimations.

Measurement of lipid peroxidation (LPO):

The extent of lipid peroxidation in the brain was determined quantitatively by performing the method as described by (21). The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using Spectrophotometer.

Superoxide Dismutase Activity (SOD):

Superoxide Dismutase activity in the brain was determined using photo oxidation of o-dianisidine sensitized by riboflavin method of (22). The change in absorbance was recorded for 4 min at 460 nm using Spectrophotometer.

Catalase activity:

Catalase activity was assessed by the method of (23), based on the ability of catalase to oxidize hydrogen peroxide. The change in absorbance was recorded for 3 min at 1 min interval at 240 nm using Spectrophotometer.

Protein estimation:

Total protein content was measured by (24). Bovine serum albumin was used as standard.

RESULTS AND OBSERVATION:

Sperm parameters:

Table I:

In the streptozotocin induced Type I diabetic rats sperm count, sperm motility and sperm viability significantly ($p<0.05$) decreased in diabetic control group when compared with normal control group. Treatment with the standard drug (Insulin 3 IU/kg) and Hesperidin (25mg/kg and 50mg/kg) sperm count, sperm motility and sperm viability were significantly ($p<0.05$) increased when compared with diabetic control.

GROUPS	NORMAL CONTROL	DIABETIC CONTROL	DIABETES + INSULIN	DIABETES + INSULIN + HESPERIDINE 25 mg/kg	DIABETES + INSULIN + HESPERIDINE 50 mg/kg
SPERM COUNT	68.33±2.6	44.66±4.05[b]	49.0±2.08	64.33±1.7[d]	66.33±2.33[d]
SPERM MOTILITY	81.33±1.45	49.66±0.33[b]	54.33±1.76	79.00±5.7[d]	80.00±0.33[d]
SPERM VIABILITY	77.33±1.20	39.00±2.08[b]	44.002.08	64.00±2.08[d]	66.33±2.186[d]

Data are expressed as mean of results in 6 rats ±SEM. [a]$P<0.0001$ and [b]$P<0.001$ between normal control and diabetic control: and [c]$p<0.0001$, [d]$p< 0.001$ between diabetic control and treated groups

ANTIOXIDANT ACTIVITY:

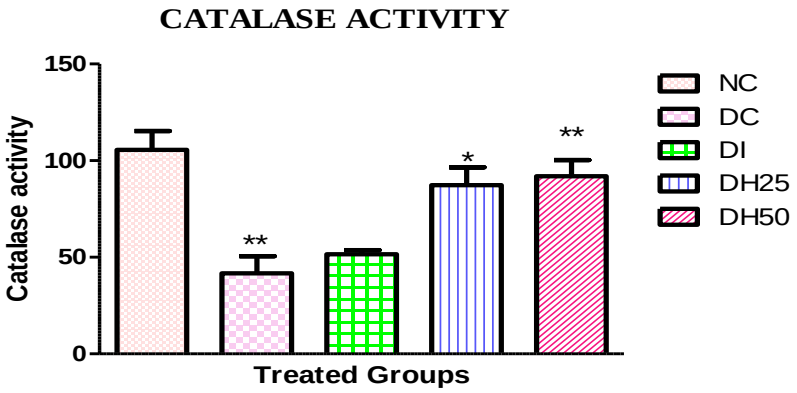
The results showed that lipids of the Type I diabetic rats are vulnerable to peroxidation due to the increased oxidative stress during diabetes ($P<0.05$). Standard drug insulin and the treatment drug i.e. Hesperidin were all able to reverse the altered LPO damage. The antioxidant enzymes CAT and SOD activities were determined in the testes of diabetic and treated rats and it was compared with control groups. In diabetic treated rats there was a decrease in SOD and CAT activities. The treatment with the standard drug insulin (3 IU/kg) and the treatment drug i.e. Hesperidin (25mg/kg and 50mg/kg) significantly ($p<0.05$) normalized the altered antioxidant enzymes levels of liver, occurred due to Type I diabetes.

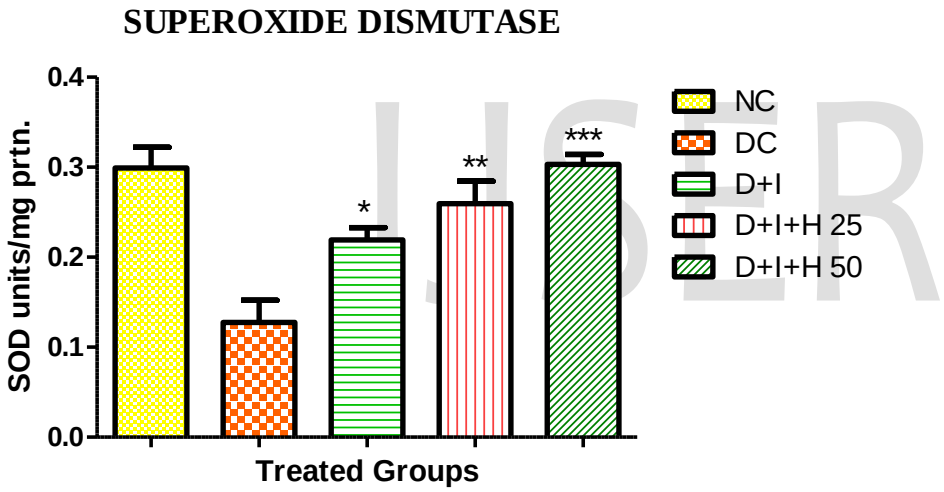
Table II:

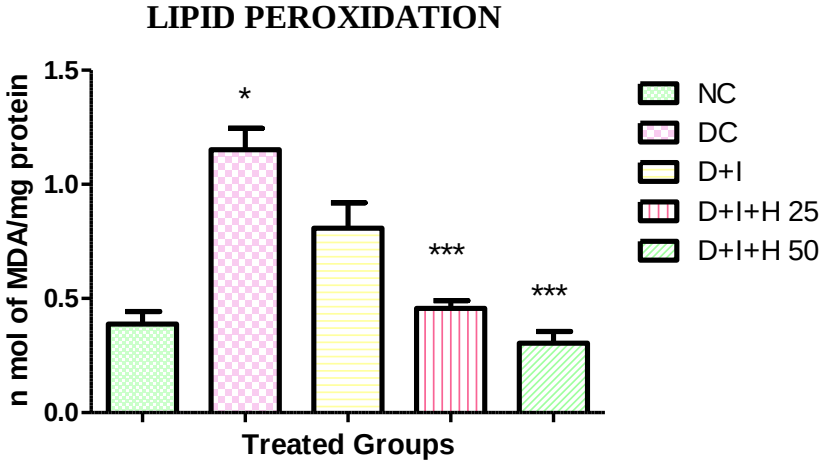
The results are presented as mean ± SEM (n = 6) of increase in baseline (%). Statistical significance was calculated by ANOVA followed by Bonferroni's test. a = $p < 0.001$ compared with vehicle treated control group.

GROUPS	NORMAL CONTROL	DIABETIC CONTROL	DIABETES + INSULIN	DIABETES + INSULIN + HESPERIDINE 25 mg/kg	DIABETES + INSULIN + HESPERIDINE 50 mg/kg
CATALASE	105.5±9.81	41.7±8.81[a]	51.5±2.14	87.35±9.2[c]	91.89±8.43[d]
SOD	0.29 ±0.02	0.12±0.02	0.21±0.01[c]	0.25±0.02[d]	0.30±0.01[b]
LPO	0.38±0.05	1.75±0.09[b]	0.8±0.11	0.45±0.3[e]	0.30±0.05[e]

Data are expressed as mean of results in 6 rats ±SEM. [a]$P<0.01$ between normal control and diabetic control: and [b]$P<0.05$, [c]$p<0.05$, [d]P<0.01, [e]P<0.001 between diabetic control andtreated groups.







Histopathology of testis:

Histopathological examination of testes was done using Hemotoxylene and eosin stain. In diabetic control group there was an inflammation and disturbed testicular architecture when compared with normal control group as shown in the Fig B. Even in insulin treated groups testicular architecture was in disturbed condition, whereas in treated groups (Hesperidin 25mg/kg and 50mg/Kg) testicular architecture largely remained intact.

Fig: A

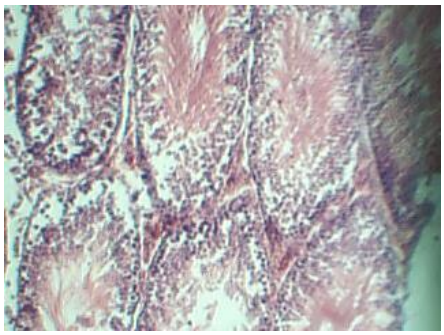


Fig: B

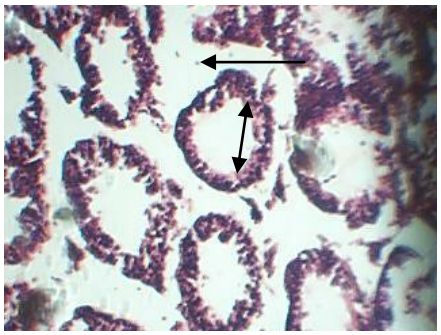


Fig: C

Fig: D

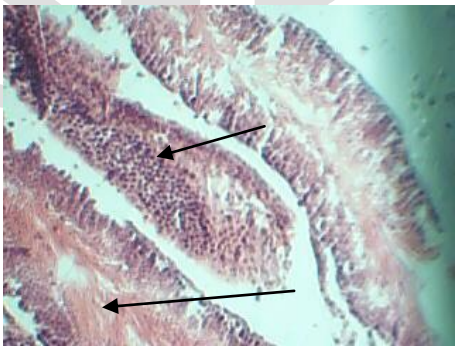


Fig: E

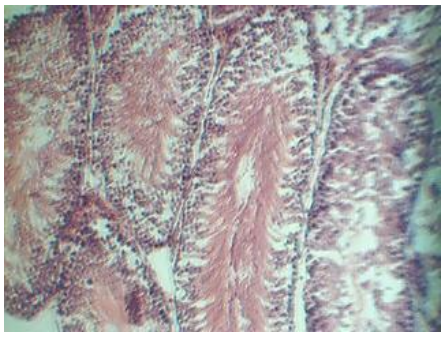


Plate photograph showing:

(A) Testis of a control normal rat showing normal seminiferous tubules (H&E 100),

(B) Testis of a control diabetic rat showing moderate degeneration of spermatogenic cells **double headed arrow** and diffuse edema of interstitial cells **single headed arrow** (H&E 100).

(C) Testis of a diabetic rat treated with insulin showing moderate degeneration of spermatogenic cells **double headed arrow** and diffuse edema of interstitial cells **single headed arrow** (H&E 100).

(D) Testis of a diabetic rat given orally 50 mg/kg of Hesperidin watery extract showing mild degenerative changes of spermatogenic cells **large arrow** and localized edema of interstitial cell **small arrow** (H&E 100).

(E) Testis of a diabetic rat given orally 25 mg/kg of Hesperidin showing normal seminiferous tubules (H&E 100).

DISCUSSION:

Incidence of diabetes mellitus is a rising worldwide and will inevitably result in an increased prevalence in men of reproductive age. It is well known that diabetes mellitus has been related with sexual dysfunction. Infertility is already a major health problem in both the developed and developing countries with up to one in six couples requiring specialist investigation or treatment for its prevention (25, 26). In this aspect, Penson et al. (2009) mentioned that male sexual dysfunction is a common complication of diabetes and the erectile dysfunction, impotence, orgasmic dysfunction, altered ejaculation and decreased libido are highly prevalent in men with chronic diabetes.(27) The ability of a sperm to maintain membrane integrity is critical for survival. A correct approach in exploring sperm oxidative stress in DM patients may start by an initial direct analysis of ROS production in basal and stimulated condition (28).

The patients with diabetes had higher rates of sexual dysfunction than the non-diabetic patients (29). On the other hand, Sandra et al. 2008 reported that diabetes causes neuropathy and vascular insufficiency that may be associated with sexual dysfunction in men. (30) Oxidative stress plays a major pathogenic role in the occurrence of secondary complications in diabetes mellitus (8). These complications can be maintained by the use of flavonoids in the diet.

Flavonoids are also recommended for their antioxidant properties. The antioxidant property of a flavonoid is determined by its structure and particularly its ability to donate a hydrogen ion to the peroxy radical produced as a result of lipid peroxidation (31,32). In diabetes, testicular dysfunction may be temporary or permanent depending on the degree and duration of the disease. Thus, this disease reduces luteinizing hormone (LH) in serum, which is responsible for normal Leydig cell function (33,34,35). This increase in sperm motility of experimental groups in comparison to control group could be due to the protective effect of Hesperidin administration. These protective effects are reflected by the decrease of malonaldehyde (LPO) level and restoration of Super oxide Dismutase (SOD) and Catalase (CAT) activities. In the present study, the different doses of Hesperidin were considered, like 25 mg/kg, (p.o) and 50 mg/kg, (p.o.). In this study 25 mg/kg (p.o.) has shown a dose dependent increase in sperm count, sperm motility, sperm viability and data from Histopathological studies confirms this claim. In a recent study, the influence of quercetin (flavonoid) was evaluated on the spermatogenesis of STZ-induced diabetes in male rats.(36) Role of antioxidant supplementation (a mixture of vitamins E and C and α-lipoic acid) on testicular germ cell apoptosis of STZ-induced diabetic rats was already evaluated. (37). our studies on hesperidin suggest greater role of bioflavonoids in the management of male infertility.

CONCLUSION:

The study indicated that streptozotocin induced diabetic mellitus in rats reduced the sperm count, sperm motility, sperm viability and causes histological changes in testis along with the increased oxidative stress. Hesperidin treatment to the diabetic animals decreased the abnormalities in

sperm parameters and enhanced the levels of SOD, CAT and reduced the levels of LPO at a dose of 25 mg/kg and 50mg/kg..

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