Influence of each hesperidin and insulin on diabetes-induced testicular alterations in adult albino rats

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SUMMARY

Diabetes mellitus is a chronic metabolic disorder that adversely affects male reproductive organs, resulting in infertility due to testicular atrophy. Hyperglycemia and oxidative stress are considered the main factors implicated in the pathogenesis of diabetic complication. Hesperidin is a natural flavonoid with wide pharmacological effects such as hypoglycemic, antioxidant and anti-inflammatory. This work aims to study the ability of hesperidin to counteract diabetes-induced testicular alterations. 50 rats were used in this experiment, divided equally and randomly into five groups: control C, diabetic DM, diabetic +hesperidin DM+H, diabetic +insulin DM+In and hesperidin group H. At the end of the experiment, the rats were sacrificed and evaluated for body weight, blood sugar level, testicular weight, histopathological, immunohistochemistry, morphometric analyses, and spermatozoa analysis. Results revealed that DM significantly reduced the body and testicular weights, produced drastic histopathological, morphometric adverse changes of the testicular tissues and morphological abnormalities of the spermatozoa. Hesperidin produced valuable hypoglycemic effect, increased the body and testicular weights, ameliorated the histopathological changes, restored normal germinal epithelium and spermatogenesis, restored morphometric parameters and significantly decreased the morphological abnormalities of the spermatozoa caused by DM. Insulin could improve some parameters that were adversely changed by DM, but less than the hesperidin. We conclude that treatment with hesperidin appeared to be more effective in counteracting the toxic effects of diabetes on testes than insulin.

Key words: Diabetes – Testis – Hesperidin – Insulin – Albino rats

INTRODUCTION

Diabetes mellitus is associated with reproductive impairment in both men and women. Its impact on reproduction can be profound, by a decrease in fertility and increase in reproductive failures (Ramalho-Santos et al., 2008). Male reproductive functions can be affected at multiple levels, including increased apoptosis in testicular germ cells, altered spermatogenesis, variation in sperm quality and quantity, decreased testicular weight, and ejaculatory dysfunction and reduced libido (Ricci et al., 2009; Jain and Jangir, 2014). Men with DM have low testosterone levels, associated with decreased luteinizing hormone and follicle-stimulating hormone concentrations. Therefore, diabetes-induced reproductive dysfunction is an important challenge (Ebong et al., 2014). Many articles explained that diabetic complications are due to hyperglycemia and overproduction of reactive oxygen species (ROS) that exceeds natural antioxidant defenses of the body, resulting in cell apoptosis (Ghlissi et al., 2012). Both testicular and sperm cells have increased susceptibility to free radical damage due to higher levels of

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polyunsaturated fatty acids, low oxygen tension and lack of antioxidant defense mechanisms (Idris et al., 2012). Many natural products have hypoglycemic activity and significant antioxidant capacity (Ebong et al., 2014).

Intake of citrus fruits and their constituting flavonoids can reduce the risk of certain chronic diseases and increase survival (Jasmin and Jaitak, 2019). Hesperidin is the predominant flavonoid in lemons and oranges. Hesperidin had a wide range of biological effects including hypolipidemic, antioxidant, anti-inflammatory, hypoglycemic, anticarcinogenic activities and has anti-apoptotic efficacy (Elshazly et al., 2018). Hesperidin showed significant antioxidant potential in terms of scavenging free radicals produced by various in vitro assays. Hesperidin showed optimum protection against free-radical-induced cellular damage (Kalpana et al., 2009). Moreover, studies have shown that hesperidin is nontoxic and causes no allergic reactions in male or female mice (Acipayam et al., 2014). So, the present study was conducted in order to compare the effects of hesperidin and insulin in counteracting diabetes-induced testicular alterations in adult albino rats.

MATERIAL AND METHODS

Animals

Fifty adult male Sprague Dawley albino rats weighing 200-250 g were obtained from the animal house of Faculty Veterinary Medicine, Suez Canal University. The rats were acclimatized two weeks before drug administration, housed in a separate cage (10 rats each). The experiment was performed at the Animal and Experimental House, Anatomy department, Faculty of Medicine, Suez Canal University. All experiments were carried out in accordance with the guidelines of the Institutional Animals Ethics Committee of Suez Canal University.

Chemicals

Streptozotocin (STZ) and Insulin were purchased from Sigma pharmaceutical, Egypt. Hesperidin was purchased from Sigma Chemical, Co., St. Louis, Mo., USA

Experimental design

The rats were randomly divided into five groups (10 rats each), as follows:

- **Group I**: Control group **(C)**, which was subdivided into:
  Group IA: rats received nothing and served as negative control.

  Group IB: rats received distilled water (the vehicle for hesperidin) orally, through an oral gavage, at a dose of 200 mg/kg/day for 10 consecutive days and served as positive control I (Arafa et al., 2009).

  Group IC: rats received citrate buffer (the vehicle for STZ) intraperitoneally at a dose of 40 mg/kg just once and served as positive control II (Kassab et al., 2019; Ricci et al., 2009).
- **Group II**: Diabetes-induced group **(DM)**, rats received STZ dissolved in citrate buffer intraperitoneally at a dose of 40 mg/kg just once. Seven days after STZ injection, rats were screened for fasting blood glucose levels (through blood obtained from a puncture of the tail vein). The rats having fasting blood glucose levels higher than 200 mg/dl were selected for experimentation (Kassab et al., 2019).
- **Group III**: Diabetes-induced group treated with hesperidin **(DM+H)**. Diabetic rats received hesperidin dissolved in distilled water orally, through an oral gavage, at a dose of 200 mg/kg/day for 10 consecutive days once hyperglycemia was confirmed (Arafa et al., 2009)**.**
- **Group IV**: Diabetes-induced group treated with insulin **(DM+In).** Diabetic rats received insulin subcutaneously at a dose of 15 IU/kg daily for 10 consecutive days, once hyperglycemia was confirmed (Moir and Zammit, 1994).
- **Group V**: Hesperidin-treated group **(H)**. Rats received hesperidin dissolved in distilled water orally, through an oral gavage, at a dose of 200 mg/kg/day for 10 consecutive days (Arafa et al., 2009).

Animals weighed daily, and drug doses were adjusted accordingly. Rats were sacrificed 24 hours after the last dose of drugs. Testes were extracted, weighed, fixed in Bouin's solution,

embedded in paraffin and sectioned at 5 µm thickness (Mahmoud et al., 2014). Left testes were used to obtain testicular weight, processed for histopathological staining, immunohistochemical and morphometric measurements.

Immunohistochemistry

- Proliferating cell nuclear antigen (PCNA): Specific monoclonal anti- PCNA antibody was used to estimate the degree of cell proliferation. Positive PCNA labeling was detected in cells located at or near the basement membrane of the seminiferous tubules (Kanter et al., 2012).
- B cell leukemia/lymphoma-2 (Bcl-2): Bcl-2 is an anti-apoptotic protein. It expressed in most tubular cells with preferential expression in cytoplasm of the spermatids close to the luminal surface (Oldereid et al., 2001).

Morphometry

The seminiferous tubules' slides were examined at x400 magnification, captured using a light microscope coupled to a digital camera (Olympus, Japan). Thirty seminiferous tubules were randomly chosen and analyzed with ImageJ software (Schneider et al., 2012; Mahmoud et al., 2014; Kianifard et al., 2011). H&E-stained sections were used for measuring thickness of tunica albuginea, diameter of the seminiferous tubules, thickness of the basement membrane of the seminiferous tubules and seminiferous epithelium height (µm). PCNA-stained sections were analyzed for measuring the number of proliferating nuclei (Kanter et al., 2012). Bcl-2-stained sections of the testis were analyzed for measuring percentage area positively stained with Bcl-2 protein (Schiller et al., 2002).

Preparation of rat sperms

Spermatozoa were collected from rats' left cauda epididymis, according to methods described by (Suresh et al., 2010).

Statistical analysis

Results were analyzed using the Statistical Package of Social Science (SPSS) computer software, version 21. All data were reported as mean ± standard deviation (SD). To evaluate significant differences, the comparison of means between each two experimental groups was done by One-way analysis of variance (ANOVA) and Post hoc Bonferroni tests. P value <0.05 was considered statistically significant and P value <0.01 was considered highly statistically significant.

RESULTS

Body weight, testicular weights and GSI

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant decrease in the final body weight, testicular weight and GSI when compared to the C group. The final body weight and testicular weight of the rats in the H group show statistically insignificant difference when compared to the C group (Table 1**)**.

Blood glucose levels

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant increase in the blood glucose levels when compared to the C group. DM+H and the DM+In groups showed a highly statistically significant decrease in the blood glucose levels when compared to the DM group (Table 2).

Table 1. Means ± SD of the body weight (initial and final), the testicular weight and GSI of the rats in the different study groups.

Parameter / Group	Body weight (gm)			Testicular weight (gm)	GSI (%)
	Initial body weight	Final body weight	Changes (gain or loss)		
C	221.40±18.88	223.60±19.28	1.90±1.52	1.51±0.23	0.67±.05
DM	218.10±16.36	181.80±8.70 ^{**a}	-36.20±13.16 ^{**a}	0.84±0.04 ^{**a}	0.45±0.01 ^{**a}
DM+H	218.20±12.00	204.40±8.47 $^{**a, **b}$	-14.30±8.56 $^{**a, **b}$	1.2±0.1 $^{**a, **b}$	0.58±0.04 $^{**a, **b, *c}$
DM+In	217.90±16.41	190.10±8.86 ^{**a}	-27.80±19.75 ^{**a}	1.0±0.08 ^{**a,*b}	0.52±0.03 $^{**a, *b}$
H	222.70±18.00	224.80±17.36 ^{**b}	2.10±1.79 ^{**b}	1.57±+0.23 ^{**b}	0.68±0.05 ^{**b}

(*) P <0.05; (**) P <0.01; [a] compared to the C group; [b] compared to the DM group; [c] compared to the DM+In group

Table 2. Means ± SD of the blood glucose levels of the rats in the different study groups.

Group	Blood glucose levels (mg/dl)
C	85.9±13.27
DM	256.30±21.26 **a
DM+H	169.70±6.79 **a, **b
DM+In	183.50±3.92 **a, **b
H	74.9±2.42 **b

(*) P <0.05; (**) P <0.01; [a] compared to the C group; [b] compared to the DM group

Histopathological results of the testicular tissues

Control group:

The rat testes of the C group showed a normal histological architecture. Homogenous tunica albuginea surrounded closely packed seminiferous tubules, enveloped with normal basement membrane and myoid cells having flat nuclei. The tubules were lined by multiple layers of distinct germinal epithelial cells with normal shape and size. Spermatogonia appeared small with oval nucleus. 1ry spermatocytes appeared as the largest cells of all spermatogenic cell series, having large vesicular nucleus. 2ry spermatocytes were of a small size and vesicular nucleus. Spermatids also appeared as small cells with vesicular nucleus. Normal spermatozoa appeared filling the lumen of the seminiferous tubules. Sertoli cells, extending between the basal lamina and the tubular lumen, were normal in size and having oval nuclei. The interstitial tissue showed clusters of Leydig cells with distinct round nucleus and pale cytoplasm. Interstitial blood vessels were of normal wall thickness and normal diameter (Fig. 1).

PCNA: PCNA-positive cells were markedly detected in basal tubular compartment, presenting a single layer of brown PCNA-positive nuclei along the basement membrane of the seminiferous tubules (Fig. 5).

Bcl-2: marked cytoplasmic expression of brown Bcl-2 protein in most tubular cells with preferential staining close to the tubular lumen (staining mainly spermatids) (Fig.6).

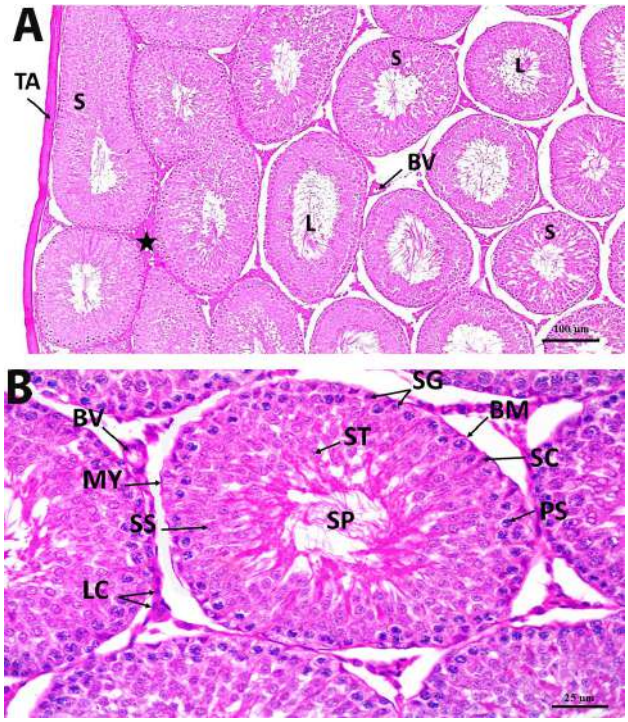

Fig. 1. Photomicrograph section in control rat testis group showing **(A)** homogenous tunica albuginea of normal thickness (TA), closely packed seminiferous tubules (S), germinal epithelium of normal height, lumen (L) full of spermatozoa, Normal interstitium (★) containing Leydig cells and normal blood vessels (BV)(H&E x 100). **(B)** normal different cells of the germinal epithelium, Spermatogonia (SG), 1ry spermatocytes (PS), 2ry spermatocytes (SS), spermatids (ST) and spermatozoa (SP). Normal sertoli cells (SC) are observed between the germinal cells. The seminiferous tubules are surrounded with a basement membrane of a normal thickness (BM) and myoid cells (MY). Interstitium shows clusters of Leydig cells (LC) and normal blood vessels (BV) (H&E x 400).

Hesperidin-treated group:

Sections of the testicular tissues of the H group showed no difference from the C group Diabetes-induced group:

The testes of the rats of the DM group showed a disrupted histological architecture. There was marked thickening of the tunica albuginea with cystic degeneration. The basement membrane surrounding the seminiferous tubules was also thickened. The seminiferous tubules decreased markedly in size. The germinal epithelium was strongly reduced in height with disrupted morphology, even atrophied in some tubules. Vacuolization was seen within the germinal epithelium. Marked decrease in the number of spermatozoa in the tubular lumen was detected. Some seminiferous tubules were even devoid completely of spermatozoa. The interstitial tissue showed marked edema and hemorrhage with dilated and congested thick-walled blood vessels. Leydig cells were remarkably damaged and decreased (Fig. 2).

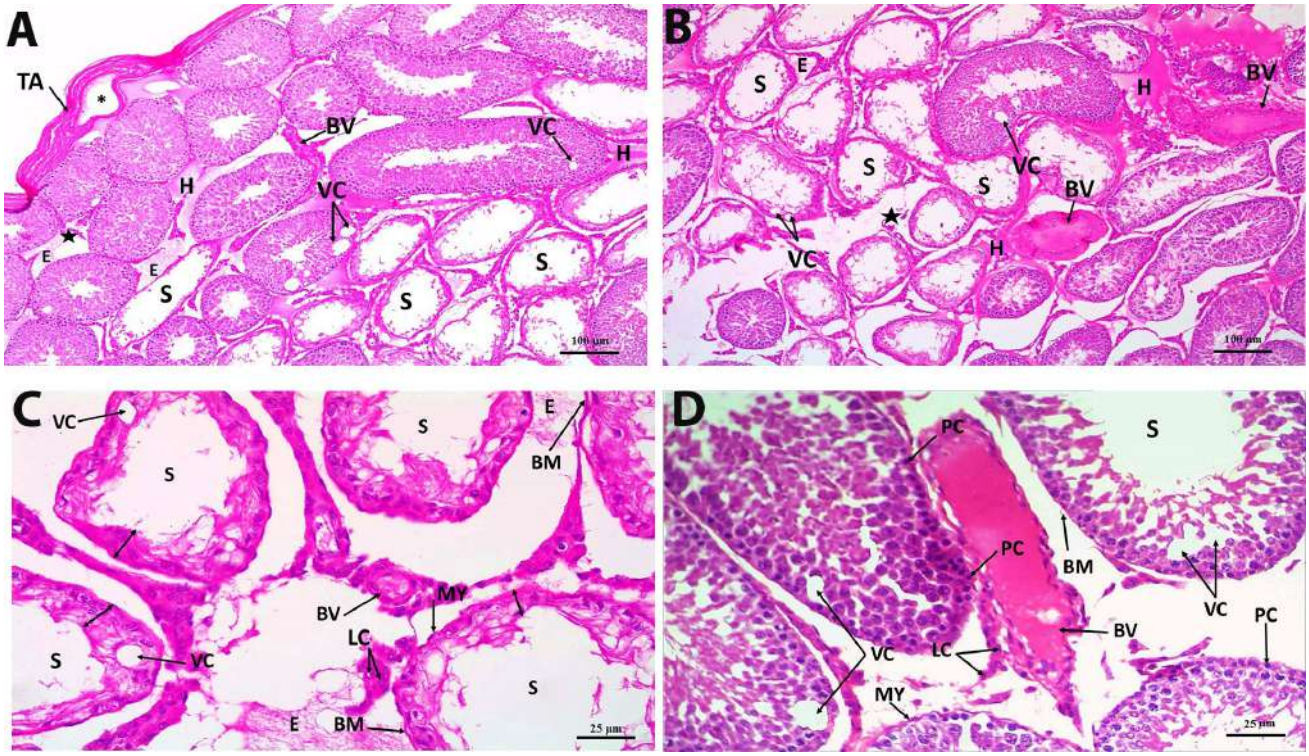

Fig. 2. Photomicrograph section in DM rat testis showing **(A& B)** thick tunica albuginea (TA) with cystic degeneration (*). Many seminiferous tubules show atrophied germinal epithelium (S), vacuolization (VC), lumen devoid of spermatozoa, Edema (E) and hemorrhage (H) are seen widely separating the tubules. Decreased interstitial mass is observed (★), dilated and congested thick-walled blood vessels (BV) (H&E x 100). **(C)** seminiferous tubules (S) having almost completely atrophied germinal epithelium (double-headed arrow), vacuolization (VC) and a lumen devoid of spermatozoa. The tubules are surrounded with detached thick basement membrane (BM) and myoid cells (MY). Leydig cells are distorted (LC), Edema (E), dilated congested thick-walled blood vessels (BV) (H&E x 400). **(D)** seminiferous tubule (S) with a lumen devoid of spermatozoa. The epithelium of other tubules shows pyknotic cells (PC). Vacuolization (VC) is found within the germinal epithelium. Detached basement membrane (BM) and myoid cells (MY) are seen surrounding the seminiferous tubules. Leydig cells are distorted (LC). Dilated congested, thick-walled blood vessel (BV) (H&E x 400).

PCNA: The number of brown PCNA-positive cells was more markedly decreased in the DM group than in the C group, even absent in some seminiferous tubules (Fig. 5).

Bcl-2: Testicular tissues obtained from the diabetic rats showed a weak expression of brown Bcl-2 protein in comparison to the C group (Fig. 6).

Diabetes-induced group treated with hesperidin:

Administration of hesperidin to the diabetic rats resulted in restoration of a near-normal morphology of the testicular tissues. The tunica albuginea was of homogenous morphology and appeared thinner than that observed in the DM group. The basal lamina surrounding the seminiferous tubules also appeared thinner when compared to that of the DM group. The germinal epithelium showed marked increased height when compared to the DM group. Almost all cell types of the germinal epithelium were noticed lining the seminiferous tubules. Spermatozoa were filling the tubular lumen. However, some vacuolization was still seen within the germinal epithelium. The interstitial tissue showed a decrease in the edema and hemorrhage, and the blood vessels were much less congested with thinner walls than those observed in the DM group. Leydig cells were increased (Fig. 3).

PCNA: There was a moderate increase in the expression of brown PCNA-positive cells in the testicular tissues of the DM+H group in comparison to the DM group (Fig. 5).

Bcl-2: There was a moderate increase in brown Bcl-2 protein expression in the testicular cells of the DM+H group in comparison to the DM group **(**Fig. 6).

Diabetes-induced group treated with insulin:

Testicular tissues of diabetic rats treated with insulin showed some improvement when compared to that of DM group. The tunica albuginea appeared thinner, although sub-capsular hemorrhage was observed. Many

seminiferous tubules showed increased diameter and increased germinal epithelial height with restoration of the different types of cells; other tubules were lined with disorganized epithelium, devoid of spermatozoa and showed degenerative changes. Vacuolization was still seen within the germinal epithelium. Some interstitial edema and hemorrhage were still observed. The interstitial blood vessels showed congestion and thick walls. Increased number of Leydig cells was observed (Fig. 4).

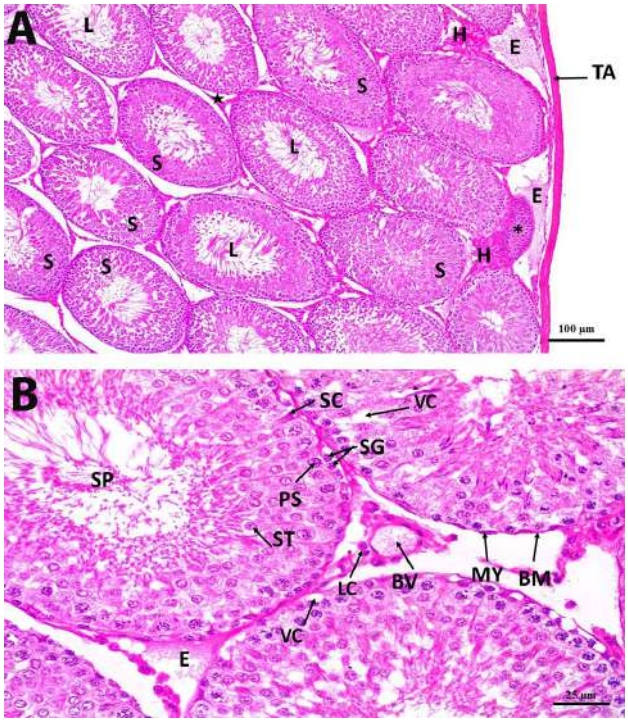

Fig. 3. Photomicrograph section in DM+H rat testis showing **(A)** homogenous tunica albuginea of an almost normal thickness (TA), closely packed seminiferous tubules (S) with regeneration of their germinal epithelium. The tubular lumen (L) is filled with spermatozoa. Degenerated seminiferous tubule is observed (*). Increase in the interstitial mass, containing Leydig cells, (★), edema (E) and hemorrhage (H) are still observed (H&E x 100). **(B)** The seminiferous tubules are surrounded with a basement membrane of a near-normal thickness (BM) and lined with almost regular germinal epithelium containing different types of cells. Spermatogonia (SG), 1ry spermatocytes (PS), spermatid (ST), spermatozoa (SP) and Sertoli cells (SC). Myoid cells are also seen surrounding the tubules (MY). Vacuolization (VC) is still found within the seminiferous tubules. Interstitium shows normal Leydig cells (LC), edema (E) and thin-walled dilated blood vessels (BV) (H&E x 400).

PCNA: There was a mild expression of brown PCNA-positive cells in the testicular tissues of the DM+In group when compared to the DM group (Fig. 5).

Bcl-2: There was a mild expression of brown Bcl-2 protein in the testicular tissues of the DM+In group when compared to the DM group (Fig. 6).

Morphometric results

1. Thickness of the tunica albuginea:

The rats of the DM, DM+H and DM+In groups showed a highly statistically significant increase in the thickness of the tunica albuginea when compared to the C group. On the other hand, the DM+H and DM+In groups showed a highly statistically significant decrease when compared to the DM group. The DM+H showed a highly statistically significant decrease in the thickness of the tunica albuginea when compared to the DM+In group. In the H group, the thickness of the tunica albuginea shows statistically insignificant difference from the C group (Table 3).

2. Tubular diameter:

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant decrease in the tubular diameter in comparison to the C group. The DM+H and DM+In groups showed a highly statistically significant increase in the tubular diameter when compared to the DM group. Moreover, the DM+H showed a highly statistically significant increase in the tubular diameter when compared to the DM+In group. The rats in the H group showed a statistically insignificant difference in the tubular diameter when compared to the C group (Table 3).

3. Germinal epithelial height:

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant decrease in the germinal epithelial height when compared to the C group. The DM+H group showed a highly statistically significant increase in the germinal epithelial height when compared to the DM group, while the DM+In group showed a statistically significant increase when compared to the DM group. The DM+H showed a highly statistically significant increase in the germinal epithelial height when compared to the DM+In group. The germinal epithelial height of the H group shows statistically insignificant difference from the C group (Table 3).

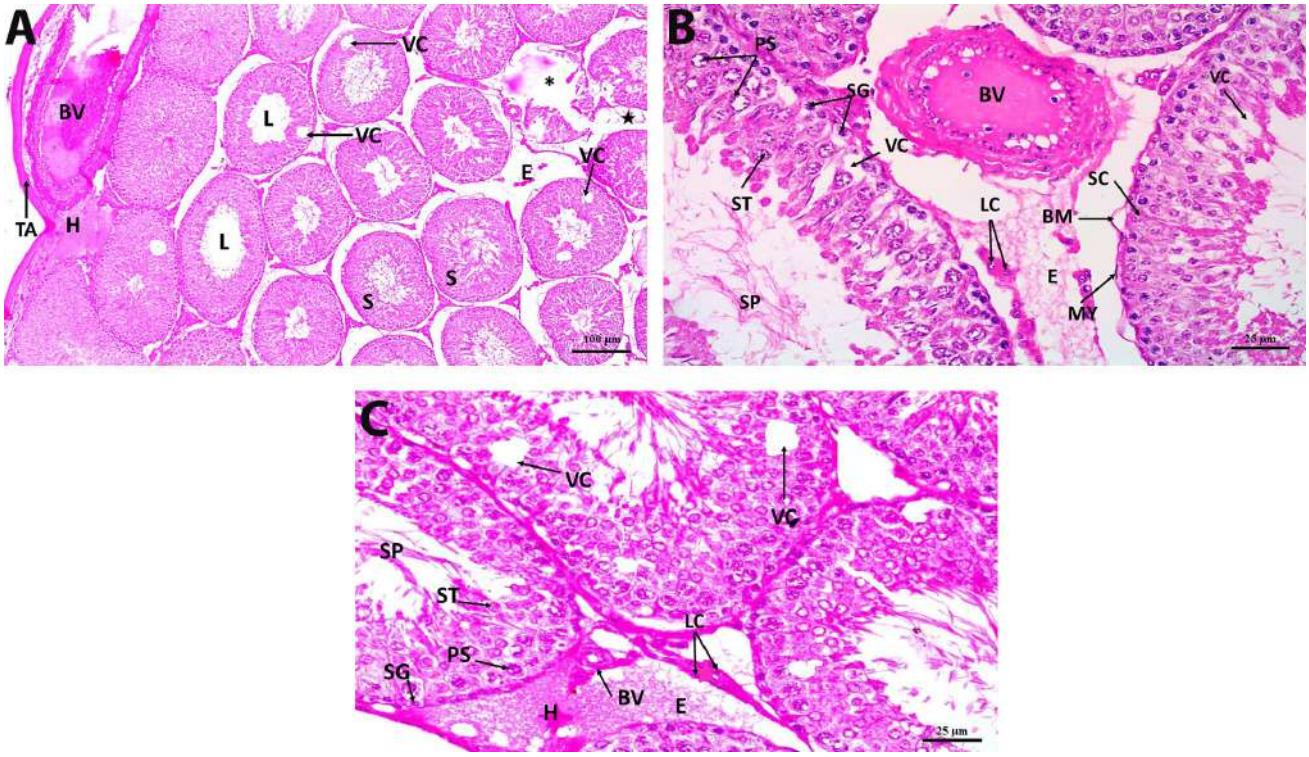

Fig. 4. Photomicrograph section in DM+In rat testis showing **(A)** thick tunica albuginea (TA), some seminiferous tubules appear normal (S), other tubules show vacuolization (VC), a lumen devoid of spermatozoa (L) and are degenerated (*). Distorted interstitial cells (★), hemorrhage (H), edema (E) are still observed, dilated, congested thick-walled blood vessels appear in the tunica vasculosa (BV) (H&E x 100). **(B)** disorganized germinal epithelial cells lining the seminiferous tubules. Spermatogonia (SG), 1ry spermatocytes (PS), spermatids (ST) and spermatozoa (SP) are seen. Sertoli cells (SC) are occasionally seen within the disrupted germinal cells. Vacuolization (VC) is still found within the seminiferous tubules. The tubules are surrounded with detached basement membrane (BM). Myoid cells (MY). Interstitium shows abnormal Leydig cells (LC), edema (E) and dilated, congested thick-walled blood vessels (BV) (H&E x 400). **(C)** disorganized germinal epithelial cells lining the seminiferous tubules. Spermatogonia (SG), 1ry spermatocytes (PS), spermatids (ST) and spermatozoa (SP) are seen. Vacuolization (VC) is still found within the seminiferous tubules. Interstitium shows some regeneration of Leydig cells (LC), edema (E), hemorrhage (H) and thick-walled blood vessels (BV) (H&E x 400).

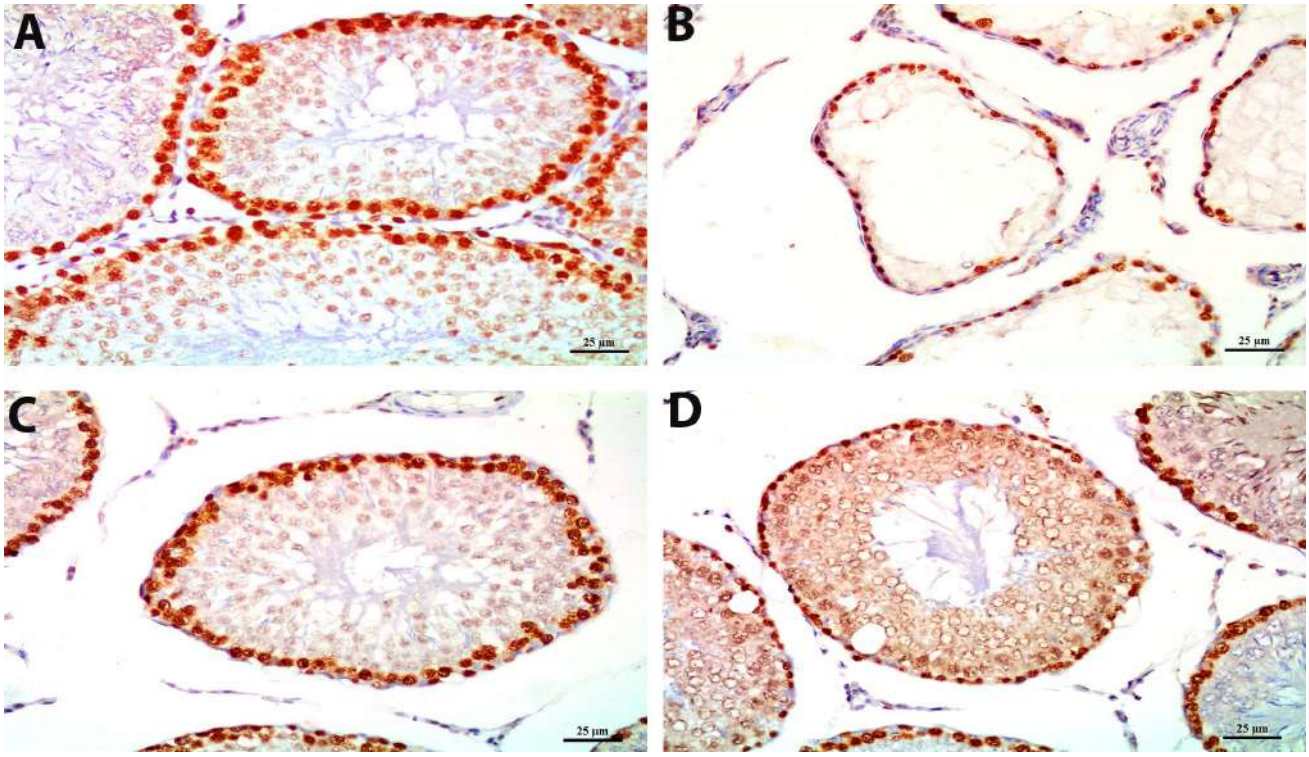

Fig. 5. A photomicrograph of a section of the rat seminiferous tubules with PCNA immuno-stain showing **(A) control group**: brown PCNA-positive nuclei that are markedly detected in the spermatogonia and early-stage spermatocytes. **(B) DM group:** marked decrease in the expression of brown PCNA-positive nuclei. **(C) DM+H group:** moderate expression of brown PCNA-positive nuclei. **(D) DM+In group:** mild expression of brown PCNA-positive nuclei (PCNA immuno-stain x 400).

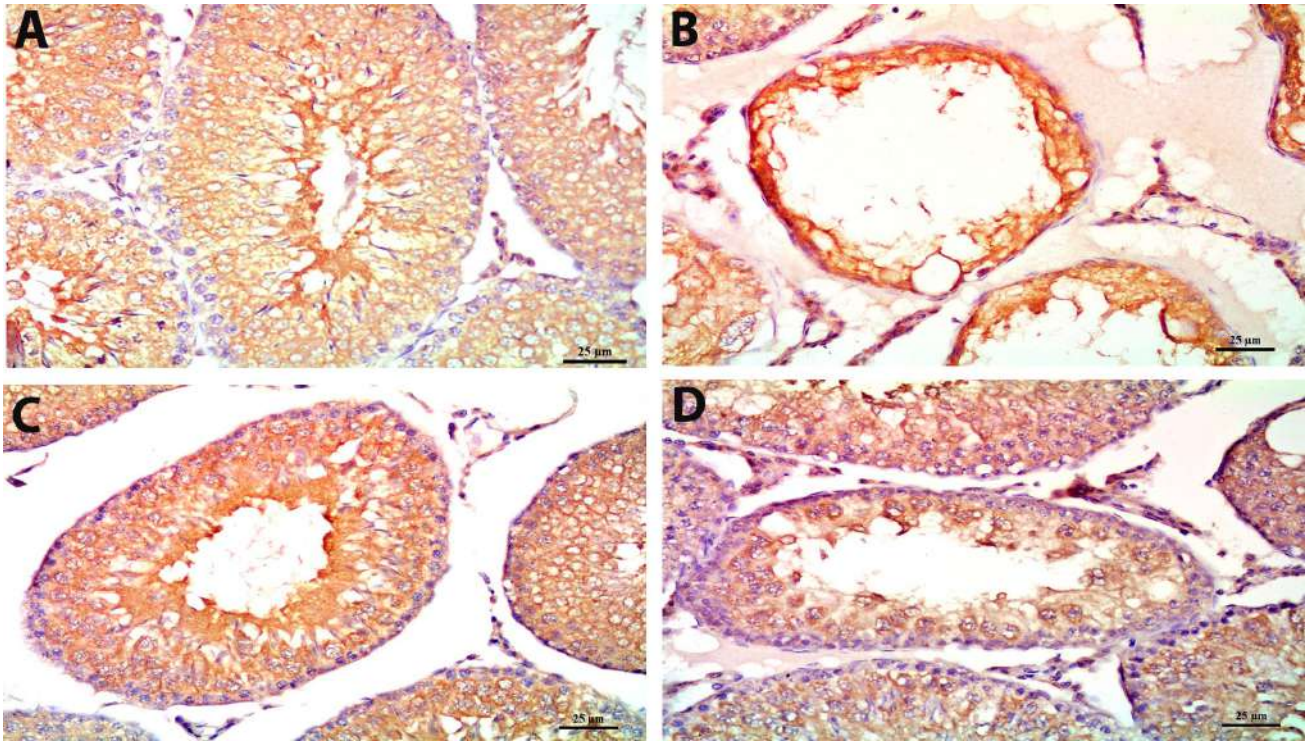

Fig. 6. A photomicrograph of a section of the rat seminiferous tubules with Bcl-2 immuno-stain showing **(A) control group:** marked expression of brown Bcl-2 protein in most tubular cells, mainly near the tubular lumen. **(B) DM group:** marked decrease in the expression of brown Bcl- 2 protein in the tubular cells. **(C) DM+H group:** moderate expression of brown Bcl-2 protein in the tubular cells. **(D) DM+In group:** mild expression of brown Bcl-2 protein in the tubular cells (Bcl-2 immuno-stain x 400).

4. Thickness of the tubular basement membrane:

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant increase in the thickness of the tubular basement membrane when compared to the C group. The DM+H and DM+In groups showed a statistically significant decrease in the thickness of the tubular basement membrane when compared to the DM group. The DM+H showed a statistically significant decrease in the thickness ofthe tubular basement membrane when compared to the DM+In group. The thickness of the tubular basement membrane of the H group show statistically insignificant change from the C group **(**Table 3**)**.

5. Number of PCNA-positive cells:

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant decrease in the number of PCNA-positive cells in comparison to the C group. The DM+H and DM+In groups showed a highly statistically significant increase in the number of PCNA-positive cells when compared to the DM group. The DM+H showed a highly statistically significant increase in the number of PCNA-positive cells when compared to the DM+In group. The number of PCNA-positive cells of the H group did not show a statistically significant difference from the C group **(**Table 3).

6. Percentage area positively stained with Bcl-2:

The rats in the DM, DM+H and DM+In groups showed a highly statistically significant decrease in the percentage area positively stained with Bcl-2 in comparison to the C group. The DM+H and DM+In groups showed a highly statistically significant increase in the percentage area when compared to the DM group. The DM+H showed a highly statistically significant increase in the percentage area when compared to the DM+In group. The percentage area positively stained with Bcl-2 of the H group show a statistically insignificant difference from the C group **(**Table 3**)**.

Sperm morphology

Normal sperm morphology was demonstrated in Fig. 7a. The normal sperm consists of head, neck, and tail. The current results showed a highly statistically significant decrease in the total

number of normal spermatozoa in the DM, DM+H and DM+In groups when compared to the C group. Head abnormalities were in the form of an absent head (Fig. 7b), a flat hook-less head **(**Fig. 7c) and an amorphous head (Fig. 7d). Neck abnormalities were mostly represented as a bent neck, either a backward bent (Fig. 7e) or a forward bent (Fig. 7f). Tail abnormalities detected were either a bent tail (Fig. 7g) or a ring tail (Fig. 7h). Also, a single sperm may exhibit multiple abnormalities (Fig. 7i) (Table 4).

DISCUSSION

DM is one of the most common endocrine metabolic disease. It affects numerous organs, system complications and dysfunction, including reproductive system. Nearly 90% of patients with DM suffer from sexual dysfunction which includes impotence, reduced libido, impaired ejaculation, and impaired semen parameters. Hyperglycemia and oxidative stress were the main factors implicated in the pathogenesis of the diabetic complications. Oxidative stress in diabetic rats causes testicular DNA damage, depletion of spermatogenic cells and delay of spermatogenesis (Long et al., 2018). The current widespread hypoglycemic agents, besides causing several side effects, have no antioxidant effect and cannot prevent the progression of the diabetic complications (Ebong et al., 2014). Thus, a regimen that can cause hypoglycemia, target oxidative stress and improve diabetic complications is required.

Table 3. Means ± SD of the morphometric parameters of the t in the different study groups.

Parameter / Group	Tunica albuginea thickness (µm)	Tubular diameter (µm)	Germinal epithelial height (µm)	Basement membrane thickness (µm)	Number of PCNA-positive nuclei	Percentage area positively stained with Bcl-2 (%)
C	79.61±0.88	597.86±3.91	210.27±3.14	2.96±0.23	70.06±3.80	49.86±0.94
DM	97.83±2.46 ^{**a}	384.05±7.86 ^{**a}	80.49±16.10 ^{**a}	8.93±0.48 ^{**a}	40.30±1.78 ^{**a}	10.69±1.84 ^{**a}
DM+H	86.27±2.09 ^{**a}, ^{**b}, ^{**c}	558.40±6.19 ^{**a}, ^{**b}, ^{**c}	186.64±1.22 ^{**a}, ^{**b}, ^{**c}	6.12±0.50 ^{**a}, ^{*b},^{*c}	59.56±3.72 ^{**a}, ^{**b}, ^{**c}	32.91±1.33 ^{**a}, ^{**b}, ^{**c}
DM+In	89.48±2.11 ^{**a}, ^{**b}	412.84±6.35 ^{**a}, ^{**b}	142.04±11.01 ^{**a},^{*b}	7.64±0.58 ^{**a}, ^{*b}	50.03±2.88 ^{**a}, ^{**b}	23.19±1.15 ^{**a}, ^{**b}
H	79.46±1.19 ^{**b}	596.06±5.30 ^{**b}	210.76±2.55 ^{*b}	2.90±0.16 ^{**b}	69.90±3.73 ^{**b}	49.62±0.90 ^{**b}

(*) P <0.05; (**) P <0.01; [a] compared to the C group; [b] compared to the DM group; [c] compared to the DM+In group

Table 4. Means ± SD of the different morphological abnormalities detected in the spermatozoa in the different study groups.

Parameter / Group	Normal	Head			Neck	Tail		Multiple
		Absent head	Hook-less	Amorphous		Bent	Ring	
C	75±4.47	2±0.7	0.2±0.44	0.00±0.00	2.6±1.34	17.8± 2.77	2.2± 1.64	0.2±0.44
		Total=2.2±0.51				**Total=20±4.41**		
DM	6.8±2.28 ^{a**}	15±3.8 ^{a**}	6.2±2.48 ^{a**}	2.2±0.83 ^{a**}	5.2±1.78	47± 4.06 ^{a**}	10± 0.7 ^{a**}	9.8±1.64 ^{a**}
		Total=23.4±7.11				**Total=57±4.76**		
DM+H	51.6±5.59 $^{a**, b**}$	5.2±1.64 $^{b**, c*}$	1.8±1.3 $^{b**, c*}$	0.6±0.54 ^{b**}	2.2±0.38 ^{b*}	26± 5.45 $^{a**, b**, c**}$	5.6±1.14 $^{a**, b*}$	4.8±1.3 $^{a**, b**}$
		Total=7.6±3.48				**Total=31.6±6.59**		
DM+In	28.6±7.75 $^{a**, b**}$	7.8±2.16 $^{a**, b**}$	3.6±2.3 ^{a**}	1.2±0.44 $^{a**, b**}$	4±2.12	38.8 ±3.89 $^{a**, b*}$	8±2.91 ^{a**}	6.8±0.83 $^{a**, b**}$
		Total=12.6±4.9				**Total=46.8±6.8**		
H	78.2±5.06 ^{b**}	3.2±1.48 ^{b**}	1.2±0.83 ^{b**}	0.00±0.00 ^{b**}	0.8±0.83 ^{b**}	15.04±4.3 ^{b**}	1.8± 0.83 ^{b**}	0.00±0.00
		Total=4.4±2.31				**Total=21.84±13.87**		

(*) P <0.05; (**) P <0.01; [a] compared to the C group; [b] compared to the DM group; [c] compared to the DM+In group

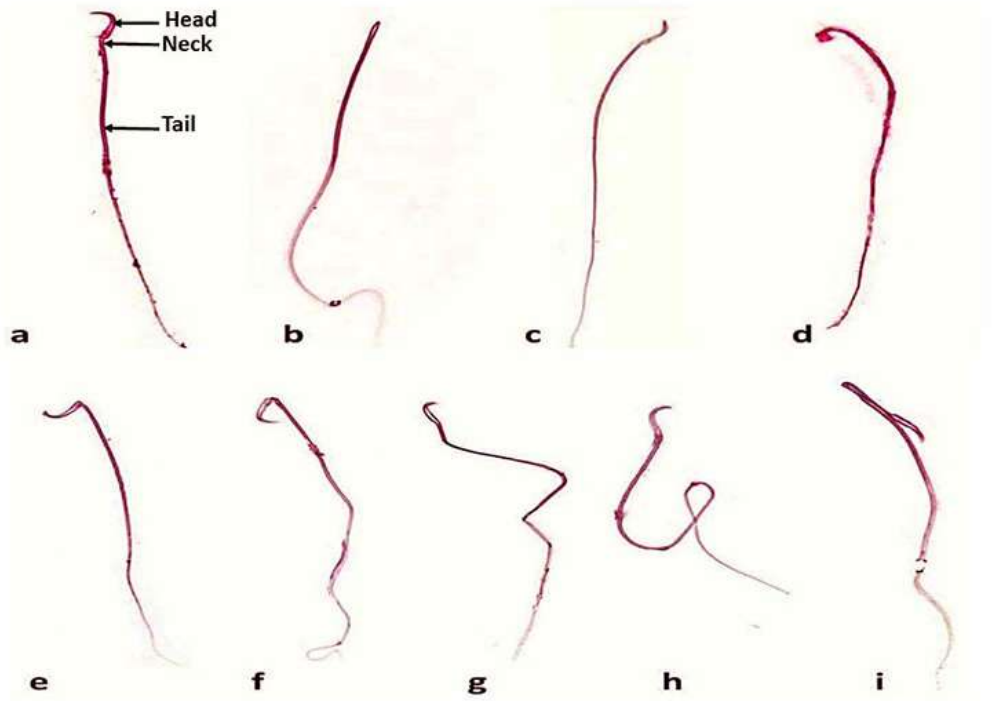

Fig. 7. A photomicrograph illustrating sperm morphology. **a:** normal (showing head, neck and tail), **b:** absent head abnormality, **c:** hook-less head abnormality, **d:** amorphous head abnormality, **e:** abnormal backward bent of the neck, **f:** abnormal forward bent of the neck, **g:** bent tail abnormality, **h:** ring tail abnormality and **i:** multiple abnormalities in a single sperm (note the hook-less head and the bent tail) (Eosin x 400).

In the current study, we compared the effect of hesperidin, a natural antioxidant, with the effect of insulin in counteracting testicular complications caused by DM.

The current results showed that the final body weight of the diabetic rats was reduced in a statistically significant way when compared to control group. Hesperidin administration increase body weight significantly while insulin did not significantly increase body weight of the rats. Weight loss of the rats observed in STZ-induced DM is not caused directly by the toxicity of STZ, but by the induction of DM (Jain and Jangir, 2014; Dkhil et al., 2016). Weight loss can be explained by loss of tissue proteins and increased muscle wasting that accompany DM (Cheng et al., 2013). STZ possibly prevents secretion of testosterone and growth hormone that results in disrupted anabolic activities, resulting in loss of weight (Long et al., 2018). The improvement of the body weight was attributed to the antioxidant effects of hesperidin and its capacity to enhance insulin secretion by pancreas, improve glucose metabolism, increase uptake of glucose by tissues and reverse most of the toxic effects of DM (Visnagri et al., 2014). Administration of exogenous insulin does not increase the rate of muscle protein synthesis (Sudha et al., 2000; Trommelen et al., 2015).

The current study showed a statistically significant decrease in the testicular weight of DM group. Administration of hesperidin caused a highly significant increase in the testicular weight. Administration of insulin increased the testicular weight significantly. Oxidative stress and hyperglycemia had a marked destructive effect on the testicular cells, causing apoptotic death, damage of the tubular epithelial lining led to marked thinning of the epithelium and atrophy. This destruction of the testicular interstitial tissue results in testicular atrophy, reduction of weight and dysfunction (Idris et al., 2012; Long et al., 2018). Administration of hesperidin increased the testicular weight significantly and ameliorated testicular weight loss (Arafa et al., 2009; Kaya et al., 2015). The protective effects of hesperidin could be ascribed to its antioxidant potentials through scavenging free radicals, improving the antioxidant defense mechanisms in testicular tissues, which in turn inhibit apoptosis, enhance

spermatogenesis, regeneration of Leydig cells and restore testicular weight (Vijaya Bharathi et al., 2015). The restoration of testicular weight with insulin treatment is through its beneficial effect on the hypothalamic-pituitary-testicular axis, thus normalizing the secretion of testosterone by Leydig cells (Schoeller et al., 2012; Dkhil et al., 2016).

GSI is used to assess the damage to the testes in relation to the body (Latif et al., 2008). In the current study, the diabetic rats showed decrease in GSI when compared to the C group. Hesperidin treatment significantly increase GSI. Insulin treatment also showed a significant increase in GSI values, but less than hesperidin group. This may be due to the fact that testes were markedly affected in diabetic rats, and that the testicular weight decreased more than the total body weight (Sangameswaran and Jayakar, 2008). Controversially, some researchers found that GSI did not change in diabetic groups. This may be due to proportional decrease in the body and testicular weights. Hesperidin increase both body weight and testicular weight, thus increasing GSI (Alluanan Adelson do Nascimento et al., 2014; Ugarte et al., 2012).

STZ in this study produced a significant elevation of the blood glucose levels. Both hesperidin and insulin significantly lowered blood glucose levels. STZ hyperglycemia is explained by beta cell destruction and alters glucose metabolism (Ahmed et al., 2012; Ugarte et al., 2012). Hesperidin effects may be through attenuation of oxidative stress, decreasing production of pro-inflammatory cytokines, increasing the sensitivity of insulin receptors, inhibiting gluconeogenesis and ameliorating pancreatic damage (Ahmed et al., 2012; Mahmoud et al., 2015). Insulin can decrease blood glucose levels through stimulating glucose uptake by skeletal muscles and adipose tissue (Ramnanan et al., 2012; Newsholme and Dimitriadis, 2001).

Concerning the current results, testicular histopathological examination of DM group showed marked thickening of the tunica albuginea and the basal lamina, and widely separated seminiferous tubules with a significant decrease in their tubular diameter. Severe loss of the germinal epithelium with abnormal arrangement and morphology of the remaining cells, widening of the tubular lumen and severe decrease in the number of spermatozoa in the tubular lumen were also seen. Damage to Leydig cells was observed. Interstitial tissues showed signs of inflammation in the form of edema, hemorrhage and dilated thick-walled blood vessels. Morphometric measurements of the testicular sections of the DM group confirmed the histopathological changes. Hesperidin administration markedly restored the structure of the testicular tissues and improved the morphometric measurements of the seminiferous tubules in diabetic rats. Insulin treatment caused mild improvement of the testicular architecture and significant improvement of the morphometric measurements. These alterations seen in the DM group could be explained by hyperglycemia which increase of cellular oxidative stress due to the overproduction of ROS and decreasing the antioxidant mechanisms. These high levels of ROS had a direct damaging effect on the tubular epithelium and Leydig cells, markedly increasing their apoptosis and atrophy, and consequently affecting sperm quantity and function (Kianifard et al., 2011; Alves et al., 2013). The diabetes-related hyperinsulinemia decreases LH levels, together with the destruction of Leydig cells, cause a decrease in the androgen biosynthesis and a decrease in the serum testosterone levels leading to a delayed abnormal spermatogenesis and a reduction in sperm output and fertility. The decreased tubular diameter is due to the atrophy of the germinal epithelium and the pressure caused by the surrounding interstitial edema and hemorrhage. The inflammatory process occurring in testis is due to the occurring oxidative stress and apoptosis, causing destruction of blood vessels, edema and hemorrhage (Ballester et al., 2004). Thick tunica albuginea and tubular basal lamina were explained by either increased collagen content, due to dysfunction of fibroblasts or glycation of the collagen. This thickness impairs blood supply to the testicular cells, thus increasing their damage and atrophy (Kianifard et al., 2011). The effects of hesperidin were attributed to its capacity to ameliorate the toxic effects of DM through its powerful antioxidant, hypoglycemic action, down-regulation of the pro-inflammatory cytokines, its beneficial effects on the capillary permeability and blood flow, it reduces diabetes-induced inflammatory edema, hemorrhage and

congested dilated blood vessels (Belhan et al., 2017). Insulin was unable to reverse the oxidative process and apoptosis in DM as it lacks the antioxidant/anti-apoptotic actions (Dkhil et al., 2016).

In the present study, PCNA-positive cells in the testicular tissues of the diabetic rats ranged from few cells in some seminiferous tubules to complete absence of positive cells in other tubules. The administration of hesperidin increased expression of PCNA-positive cells compared to DM group. The insulin-treated rats showed mild improvement in comparison to the DM group, but less than the hesperidin-treated group. The ability of hesperidin to stimulate the proliferation of cells in DM is attributed to its antioxidant/hypoglycemic, leading to enhanced cellular proliferation and survival stated by (Mahmoud and Hussein, 2014; Dkhil et al., 2016).

In this study, Bcl-2 sections of diabetic rats showed a marked decrease in the expression of the anti- apoptotic protein Bcl-2. Hesperidin markedly upregulated expression of Bcl-2 in the testicular tissues. Insulin was noticed to mildly increase Bcl-2 expression. This can be explained by increased testicular cell apoptosis accompanying DM (Jiang et al., 2015). Hesperidin provide evidence for the anti-apoptotic properties.

In the current work, sperm morphological abnormalities were increased significantly in the diabetic rats, in which tail abnormalities were the most common, followed by head abnormalities, then multiple abnormalities. Neck abnormalities were the least common. Administration of hesperidin to the diabetic rats greatly improved sperm abnormalities. Insulin treatment also lessened many sperm abnormalities. Increased abnormal sperm forms in the diabetic rats is attributed to disrupted glucose metabolism and the damaging effect of ROS to the spermatozoa. The susceptibility of the spermatozoa to ROS is high, particularly in the epididymis, because spermatozoa produced in the testis are reasonably more protected by the microenvironment of SCs, but they are less protected against oxidation in the epididymis, where they are stored. Oxidative stress led to DNA damage of the sperm cells, altered membrane functions, impaired motility, and decreased fertilization capacity. As insulin cannot work as an antioxidant, some abnormalities were still seen with insulin treatment.

Conclusion

Treatment with hesperidin appeared to be more effective in counteracting the toxic effects of diabetes on testes than insulin.

Author contributions

All authors have been personally, equally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

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