Original Article

The Possible Protective Role of Vitamins C&E Supplemented Diet on the Testis of Adult Albino Rat with Induced–Diabetes, An Ultrastructural, Morphometrical and Biochemical Study

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ABSTRACT

Introduction: Diabetes is the commonest endocrine disease in the world. Its pathological effects extend nearly to all organs. Oxidative stress is one of the mechanisms by which diabetes causes organ damage. Diabetes leads to development and exaggeration of testicular disorders.

Aim of the Work: This study aimed to evaluate the effect of the vitamins (C&E) treatment prior to and concomitant with development of diabetes. The effects are studied histopathologically, ultrustructurally, biochemically, and morphometrically.

Material and Methods: Forty adult male albino rats were used in this study. They were divided into four equal groups: control (Group I: given food and water ad libitum); Vehicle control (Group II: given intraperitoneal injection of citrate buffer only 0.1 M, pH = 4.5); Diabetic (Group III: 40 mg/kg streptozotocin dissolved in citrate buffer injected intraperitoneally); dia-betic group with vitamin C&E (Group IV: Vitamin C & E supplemented diet was introduced for six weeks (2 weeks prior to induction of diabetes and for four weeks concomitant with diabetes). Blood testosterone level was measured. Seminiferous tubules' surface area, Johnsen-like score and basement membrane thickness image analysis were evaluated.

Results: In diabetic group, ultrastructural examination revealed a thickened and distorted basement membrane and wide intercellular spacing. Morphometrically, diabetes reduced Johnsen-like score and increased tubular surface area. Furthermore, blood testosterone level in diabetic rats was significantly decreased when compared with the control group. These alterations were ameliorated with vitamins treatment prior to and concomitant with diabetes as the basement membrane was thin, regular, and there was an increase in germinal strata.

Conclusion: The treatment with combined vitamin C & E in diabetes was effective in reducing oxidative stress and oedema of testes and significantly increased Johnsen like score. this supports the importance of nutritional administration of antioxidant Vitamins

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Key Words: Interstitial leydig cell, johnsen-like score, sertoli cell, streptozotocin, testosterone.

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INTRODUCTION

The commonest endocrine disease in the world, Type 2 diabetes mellitus, leads to cardiac, renal and nervous complications[1]. Type 2 diabetes mellitus causes many pathological effects on the male reproductive systems including hypogonadism, impaired spermatogenesis and infertility[2,3]. The increasing prevalence of diabetes is mainly caused by reduction of physical activity and consumption of fast and un-healthy food in addition to genetic susceptibility in some individuals[4].

In diabetes persistent hyperglycemia may lead to overproduction of free radicals by increasing glucose auto-oxidation resulting in disturbing oxidant antioxidant balance[5]. Germ cells and sperms are more vulnerable to free radicals damage produced by low oxygen tension, higher level of poly unsaturated fatty acid, and diminished antioxidant defense mechanism[6].

Vitamin E has shown an important protective effect on sperms and hematology in diabetic rats under glibenclamide treatment, suggesting its therapeutic use in the management of diabetes mellitus in male[7]. Vitamin E proved to be protective against many toxic materials[8]. Vitamin C acts as a reducing agent in many enzymatic reactions. It can react with almost all other oxidized free radicals, so it is considered an antioxidant. Vitamin C plays a vital role in collagen synthesis[9]. Moreover, vitamin C is very important in the fertility of human and animals as it maintains spermatogenesis, preserves the viability of sperms, stops sperm agglutination, and increases the testosterone in serum[10].

Streptozotocin-induced diabetic rat is considered a good model of beta-cell damage through glucose toxicity[11,12]. The streptozotocin does not directly affect the structure of testicular tissue of sterptozotocin – induced diabetic rats[13].

In diabetes, the levels of vitamins C and E are significantly reduced in plasma and tissues[14]. Vitamins C and E act as antioxidants either alone or in mixture and co-administration of both vitamins is more effective[15]. The plasma vitamin E level is reported to be reduced in diabetic patients more than that of healthy individuals[16].

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Many studies have demonstrated the protective antioxidant effects of vitamins E and C on diabetes, but they did not throw more light on the ultrastructural and morphometric changes and the possible prophylactic effect of vitamins enriched diet and good healthy nutrition in diabetes. Hence, the aim of this work was to assess the prophylactic role of co-administration of both vitamins C & E against ultrastructural, histopathological, biochemical, and morphometric changes in the testis induced by diabetes.

MATERIAL AND METHODS

Chemicals

Streptozotocin (STZ): was purchased from Sigma-Aldrich chemical Co. (St. Louis, MO., USA). Vitamin C (ascorbic acid) and Vitamin E (di-α tocopherol acetate) were purchased from Cairo Company Lab. for Chemical and Medical Trading, Zagazig, Egypt.

Animals

Forty adult male albino rats (around 7 months age) were supplied from Zagazig Scientific & Medical Research Center (ZSMRC), Zagazig University, AlSharkia, Egypt. Their weighs at the beginning of the experiment ranged between 150-250g. Rats were housed in plastic cages and maintained at room temperature (21-23 C) with 12hour light/dark cycles. Rats were kept on a standard diet and free intake of water ad libitum following IACUC instructions with approval number ZU-IACUC/F/36/2019. They were bred for two weeks to adapt to the experimental site.

Experimental Design

The rats were randomly divided into four equal groups (10 for each).

Group I (control group): kept on standard diet (6% fat in the form of butter, 20% protein in the form of casein, 21% water and 53% carbohydrates in the form of sucrose sugar[18]).

Group II (vehicle group) allowed for standard diet and received intra peritoneal injection of citrate buffer only (0.1 M, pH = 4.5)[17].

Group III (Diabetic group) induction of diabetes was performed by intraperitoneal injection with a single dose of STZ 40 mg/kg body weight dissolved in citrate buffer[19]. The level of fasting blood glucose was measured 48 hours after the injection of STZ, using a one-touch basic pulse glucometer (Lifescan, Inc., Milpitas, California, USA), the blood glucose level from 250 mg/dl and higher was considered diabetic[20].

Group IV (Diabetic and vitamins C&E group [VCE group] Rats were fed with VCE (Vitamins C and E) supplement-ed diet for six weeks (2 weeks prior to induction of diabetes and four weeks after induction). The VCE-supplemented diet contained a dose of vitamin C (40mg/kg body weight) and vitamin E (150mg/kg body weight)[17] administrated orally by a gavage tube as a single daily dose. Food consumption of all groups was properly observed throughout the experiment.

Sampling

After six weeks, by the end of the experiment, rats were weighed, and the final weight and fasting blood glucose were estimated, and both were used for statistical analysis. Rats were randomly selected from each group and animals were anaesthetized with 50 mg/kg sodium phenobarbital intra-peritoneally. Blood samples were obtained from the retroorbital venous plexuses of all rats via capillary tubes with no ethylene diamine tetra acetic acid. These were left at room temperature for 15 min to coagulate then centrifuged at 2400rpm for 20 min for serum collection[21]. For later hormonal analysis, the resultant serum was stored at −20˚C. The testes were dissected out and processed for microscopic examination.

Histopathological Evaluation

Specimens from the left testes from all animals were obtained for histopathological examination. Each testis was cut into 10 slices perpendicular to the long axis of the organ. Slices were fixed in buffered neutral formaldehyde 10 % solution and treated to make 5-µm thick paraffin sections and stained with hematoxylin and eosin (H&E)[22]. The specimens' preparation was done in Nasser institute's laboratory, while the prepared specimens were examined in the light microscopic unit, Department of Anatomy and Embryology, Faculty of Medicine, Zagazig University.

Electron microscope study

Fresh testis specimens from right testes from all animals were fixed in 1% glutaraldehyde and 4% paraformaldehyde in phosphate buffer for 1 h. Then small pieces from fresh testis specimens, (about one cubic millimeter in size) fixed in 1% osmium tetroxide for 1h, dehydrated through graded alcohol series, and embedded in epoxy resin. Ultrathin sections of testis (70–90 nm) were collected on copper grids and stained with uranyl acetate and lead citrate[23]. The specimens' preparation was done and examined in the electron microscopic unit in Faculty of Medicine, Tanta University.

Body weight and blood glucose level

During the duration of the experiment, body weight and fasting blood glucose level were determined for all animals weekly and the measures of the last week of the experiment were used for statistical analysis.

Determination of serum testosterone level

Serum free Testosterone was meas-ured via rat-specific enzyme-linked immunosorbent assay (ELISA) commercial kits of Elabsciencefi Biotechnology Inc. (Cat. No.: MBS282195, MBS764675, MBS2502190, and MBS263466, respectively, Houston, TX, USA) with the Zirkin and Chen method[24]

Morphometric study

From each section seminiferous tubule surface area/ μm2 (essentially from circular tubular cross sections) was determined by using "Leica Quin 500 C" image analyzer computer system (Leica Imaging System Ltd., Cambridge, UK). Five 10× round or nearly round non overlapped randomly selected testicular images/slides were captured for measuring tubular surface area and analyzed by using "Leica Quin 500 C" image analyzer computer system (Leica Imaging System-Ltd., Cambridge, UK). Three slides were used from each animal and five animals from each group were used. The basement membrane thickness was measured by electron microscope.

Assessment of spermatogenesis

Spermatogenesis was evaluated by using the Johnsen-like score. The Johnsen score[25] is the standard method for categorizing human spermatogenesis, and it was modified to be used in the rabbit spermatogenesis analysis. The modified Johnsen score was named the Johnsen-like score[26]. In each group, 50–100 cross sec-tions of seminiferous tubules were evaluated[27] according to standards presented in (Table 1).

Table 1: Adaptation of Johnsen like score[25] for the evaluation of spermatogenesis in rat

Score	Evaluation of spermatogenesis
10	Complete spermatogenesis with mature sperm cells
9	Some sperm cells with a disorganized epithelium
8	Few sperms (< 5 to 10) >
7	No sperms, only spermatids
6	No sperms, few spermatids (< 5 to 10)
5	No sperms, no spermatids, presence of spermatocytes
4	No sperms, no spermatids, few spermatocytes (< 5)
3	Only spermatogonia present
2	Sertoli cells only
1	No cells visualized in tubular cross section

Statistical analysis

Results were statistically analyzed using Graph Pad Prism 5 (Graph Pad Soft-ware, San Diego, USA). The obtained data were expressed as mean values ± SD and differences between groups were deter-mined by ANOVA test followed by Bon-ferroni's Multiple Comparison post-hoc test for Multiple Comparison of different groups. The difference was considered to be significant at $p < 0.05$.

RESULTS

Light microscopic examination

Light microscopic examination of the testis of the first two groups (Groups I and II) showed similar results; thus, we select Group I as the representing group in the clarification of the results. The histological structure of the testicular tissue of control rats contained many regular closely packed seminiferous tubules with narrow lumen. Seminiferous tubules were lined by stratified and well-organized germinal epithelium arranged in more than 5 layers. These tubules were seen resting on a well-developed thin basement membrane with sperms filling their lumina.

The interstitial tissues lying between the seminiferous tubules appeared scanty and the interstitial spaces were narrow (Figure 1A). The Sertoli cells were pyramidal in shape and showed cytoplasmic extensions. The spermatogonia rested on basement membrane. The seminiferous tubules were surrounded by regular thin basement membrane ensheathed by one layer of flat myoid cells surrounding the tubules. The interstitial tissues containing few Leydig cells which have eosinophilic cytoplasm. The Lumina were filled with spermatozoa (Figure 1B).

Testicular tissue stained by toluidine blue showed seminiferous tubule lined by discrete germ layers with closely packed relations between germ cells and supported by Sertoli cell with vesicular nucleus and prominent nucleolus. Round spermatids lie in an adluminal position while the elongating spermatids lie at the luminal surface. Leydig cell showed light stained cytoplasm and prominent nucleolus in between tubules. (Figure 1C)

The diabetic group (group III) showed disorganized testicular tissue. Some tubules had sloughed basement membrane; others were atrophied, while there were depletion of testicular tissue in other areas. Basement membrane was distorted. Interstitial spaces were wider and contained acidophilic substance and vacuolations (Figures 2A,B). Germinal epithelium showed few, poorly arranged layers and wide inter-cellular spaces. Interstitial tissue contained numerous thickwalled blood vessels which were surrounded by elongated darkly stained infiltrating cells and increased number of Leydig cells. In some areas, interstitial tissue markedly increased wherever it encroached and distorted adjacent basement membrane and contained numerous small darkly stained inflammatory cells (Figures 2C,D).

Tissues stained by toluidine blue confirmed the findings of blood-walls' thickening and basement membrane thickening in diabetic group. Depletion of germ cells with wide intercellular spaces and loss of testicular tissue in some areas were obvious. Giant large cell which had multiple nuclei was present (Figures 2E,F).

Diabetes and vitamins group (group IV) showed testicular tissue with preserved structure. There was a better organization of seminiferous tubules which was ensheathed by regular basement membrane. Lumina were filled with spermatozoa tails, few tubules have sloughed germinal epithelium from their basement membrane, and there was a narrower interstitial space than diabetic group. (Figure 3A). Seminiferous tubules were lined by better organized germinal epithelium. Sertoli cell rested on basement membrane. Some areas showed wide intercellular spaces. Interstitial tissue showed few Leydig cells with vesicular nuclei. (Figure 3B). These findings were confirmed in sections stained by toluidine blue

as seminiferous tubule surrounded by regular and thin basement membrane, spermatogonia with dark nuclei rested on the basement membrane, spermatozoa heads were embedded in between cells, the cells appeared packed to each other. Blood vessels had thin wall and were surrounded by few fibroblasts (Figure 3C).

Transmission Electron Microscopy

Electron microscopic examination of germinal epithelium of control groups revealed Sertoli cell which were identified by large euchromatic nucleus with prominent nucleolus, cytoplasm containing many mitochondria. Spermatocytes appeared round cells and their nuclei showed some electron dense clumps of heterochromatin and electron lucent cytoplasm. (Figure 4A). The spermatid cells were closely packed and displayed large ovoid euchromatic nuclei with homogenously distributed chromatin materials and surrounded by distinct nuclear membrane. The formation of the acrosome in the developing spermatid was well manifested. This formation started with the appearance of a large acrosomal granule within an acrosomal vesicle, which appeared adherent to the anterior pole of the nucleus, then distributed over the ante-rior pole of the nucleus to form an acrosomal cap. Their cytoplasm was lightly stained and contained vacuolated mitochondria aggregated at the periphery towards the plasma membrane (Figure 4B).

Leydig cells had oval euchromatic nucleus with marginated heterochromatin. Its cytoplasm contains numerous electron dense mitochondria and lipid droplet (Figure 4C). There were massive ultrastructural alterations of all cellular testicular tissues of the diabetic rats. Some Sertoli cells had apoptotic small nuclei with peripheral clumps of heterochromatin. The cytoplasm showed many vacuolations which presented more in apical than basal part and distended mitochondria which had electron dense membrane and electron lucent matrix and residual bodies were present. The close relation between Sertoli cell and germ cells was lost as there was wide intercellular spaces (Figure 5A).

Also in the same group, the basement membrane was markedly thickened and irregular, apoptotic spermatogonia had irregular shrunken dark nuclei, their cytoplasm was electron dense with ghost cell appearance and surrounded by irregular cell membrane. Wide intercellular spaces between cells (Figure 5B).

Some Sertoli cells had euchromatic nuclei however, the cells did not rest on basement membrane but were separated from this irregular thick basement membrane (Figure 5C). Moreover, there was disturbance in the formation of the acrosome in the developing spermatid. An acrosomal vesicle with distorted outer membrane containing small acrosomal granule was observed. It appeared detached from the atrophied nucleus. No acrosomal cap was apparent covering the hemisphere of the nucleus, its cytoplasm contained few mitochondria and multivesicular bodies. (Figure 6A). The middle pieces of spermatozoa tails had central axonemes surrounded by disorganized and distorted electron dense fibers and outer swollen mitochondrial sheath. Longitudinal sections showed head and acrosomal cap. However, some sperms lacked acrosomal cap (Figure 6B).

The interstitial Leydig cell was shrunken and showed features of degeneration. It contained heterochromatic nucleus with marginated heterochromatin. The cytoplasm revealed few disrupted mitochondria, intracellular vacuolations and few lipid droplets. Another degenerated Leydig cell has dark pyknotic nucleus with illdefined nuclear membrane, cytoplasm was dark with illdefined organelles (Figure 6C).

The diabetic rat testis treated by vitamins C&E, showed mild improvement in their structures. The Sertoli cells were lying on relatively thin and regular basement membrane. They showed homogenous euchromatic nuclei, prominent nucleoli. The cytoplasm contained moderate number of small mitochondria with no apparent cytoplasmic vacuoles. No wide intercellular spaces between Sertoli cell and adjacent spermatocytes were detected (Figure 7A). Round spermatids had rounded euchromatic nuclei with finely distributed chroma-tin, acrosomal vesicle containing acrosomal granule at one side of the nucleus and many characteristic electron lucent, marginated mitochondria were present at the periphery of the cytoplasm (Figure 7B). The middle pieces sections of spermatozoa showed in-tact central core of axoneme which was surrounded by nine electron dense fibers and outer electron dense mitochondrial sheath. The principal pieces showed intact core of axoneme surrounded by nine dense fibers and outer fibrous sheath, some middle pieces had swollen mitochondria (Figure 7C).

The interstitial Leydig cells displayed oval euchromatic nuclei with marginated heterochromatin. The cytoplasm was still electron dense and contained nu-merous electron dense mitochondria and few lipid droplets (Figure 7D).

Body weight and blood glucose level

The representation of body weight and blood glucose level were illustrated in (Table2, Figure 8). At the end of the exper-imental period (6 weeks), the tables showed a significant ($p < 0.001$) loss in body weight in both diabetic (group III) and VCE group (group IV) compared to the control groups. Moreover, there was significant difference between diabetic and VCE treated group. According to blood glucose level in the control groups (Figure 8B), there were normal blood glucose concentrations which were significantly different compared to the diabetic groups. The STZ-treated rats had an increase in the blood glucose concentrations, and the values were significantly higher ($p < 0.001$). There was no significant difference between diabetic group and VCE group where blood glucose level was still higher than control groups ($p > 0.05$) to the normal control rats (group I). On the other hand, proper observation of food consumption in all groups did not reveal any decrease in food consumption in diabetic group.

Biochemical results

One - way analysis of variance (ANOVA) test revealed that the blood testosterone level in STZ-induced diabetic rats was significantly decreased in comparison to control rats ($P < 0.05$). There was increase in testosterone level in diabetes and vitamins group but still lower than control group and statistically non-significant (Table3, Figure 9).

Assessment of spermatogenesis

The mean Johnsen-like scores of the diabetic group were highly significantly decreased in comparison to the control and VCE groups ($P < 0.001$***) (Table 3, Figure 11).

Morphometric results

One way ANOVA test and Bonferroni's Multiple Comparison post- hoc test regarding tubular surface area (TSA) parameter. There was marked increase in Di-abetic group comparing with control groups which was statistically significant between diabetic and control groups $P < 0.05$ *** , while there was marked decrease in diabetic and vitamins group comparing with diabetic group, there was statistically highly significant decrease in TSA in group IV in comparison with group III($P < 0.05$** (Table3, Figure 10). Regarding basement membrane thickness, there was statistically significant increase in thickness in diabetic group which was ameliorated (statistically significant decrease) by vitamins treatment in VCE group (Figure 12, Table3).

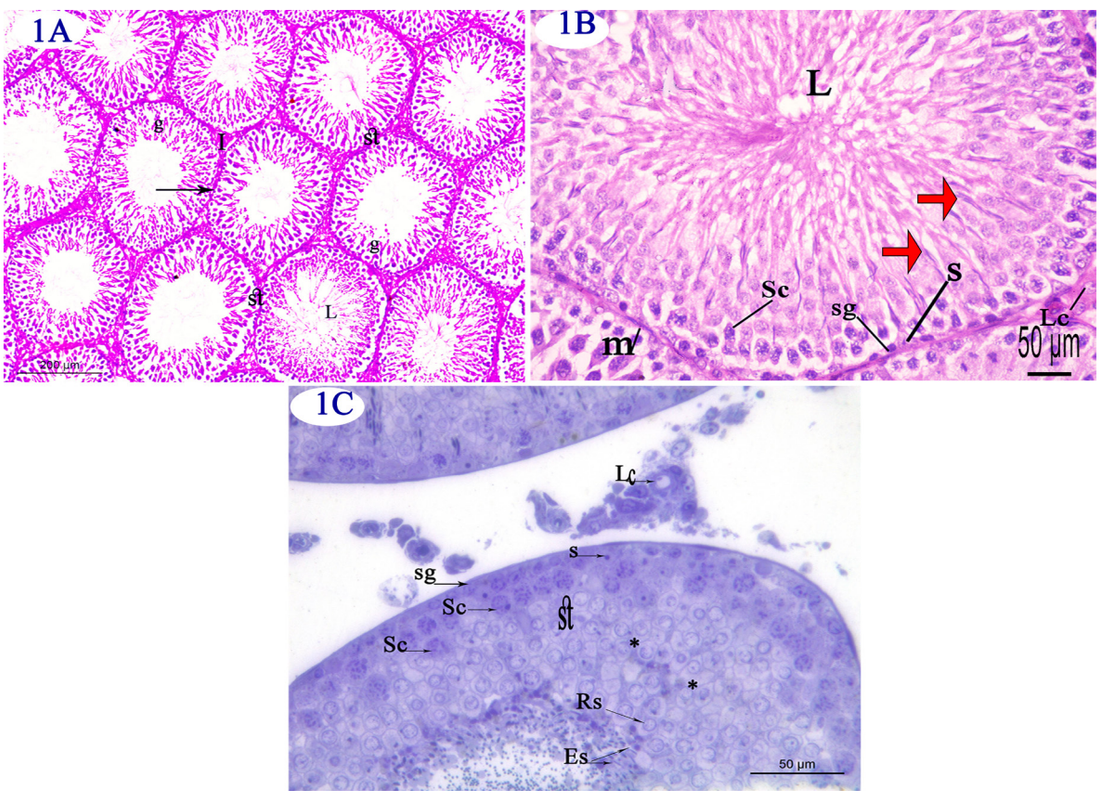

Fig. 1: Photomicrographs of sections in the testis of control groups (groups I&II). [1A]: shows closely packed seminiferous tubules (st) with narrow lumen (L). They are lined by germinal epithelium (g) resting on a thin basement membrane (arrow), the interstitial spaces (I) lying between the seminiferous tubules appears scanty. (H&E ×100) [1B]: The seminiferous tubules are ensheathed by flat myoid cells (m). They are lined by pyramidal shaped Sertoli cell (S). The Sertoli cells (S) send cytoplasmic extensions (red arrows)., spermatogonia(sg) resting on basement membrane, spermatocytes (Sc). Few interstitial Leydig cells (Lc) with eosinophilic cytoplasm are present in interstitial space, lumen(L) is filled with spermatozoa, (Johnsen-like score 10). (H&E ×400) [1C]: section of testicular tissue stained by toluidine blue seminiferous tubule (st)lined by discrete germ layers supported by the Sertoli cell (s) with vesicular nucleus and prominent nucleolus. Spermatogonia (sg) lie on the basal lamina, spermatocyte (Sc) lies midway in the epithelium, round spermatids (Rs) lie in an adluminal position, and the elongating spermatids lie at the luminal surface. Leydig cell (Lc) with light stained cytoplasm and prominent nucleolus in-between tubules. Notice closely packed relations between germ cells (astresicks) (toluidinebluex400).

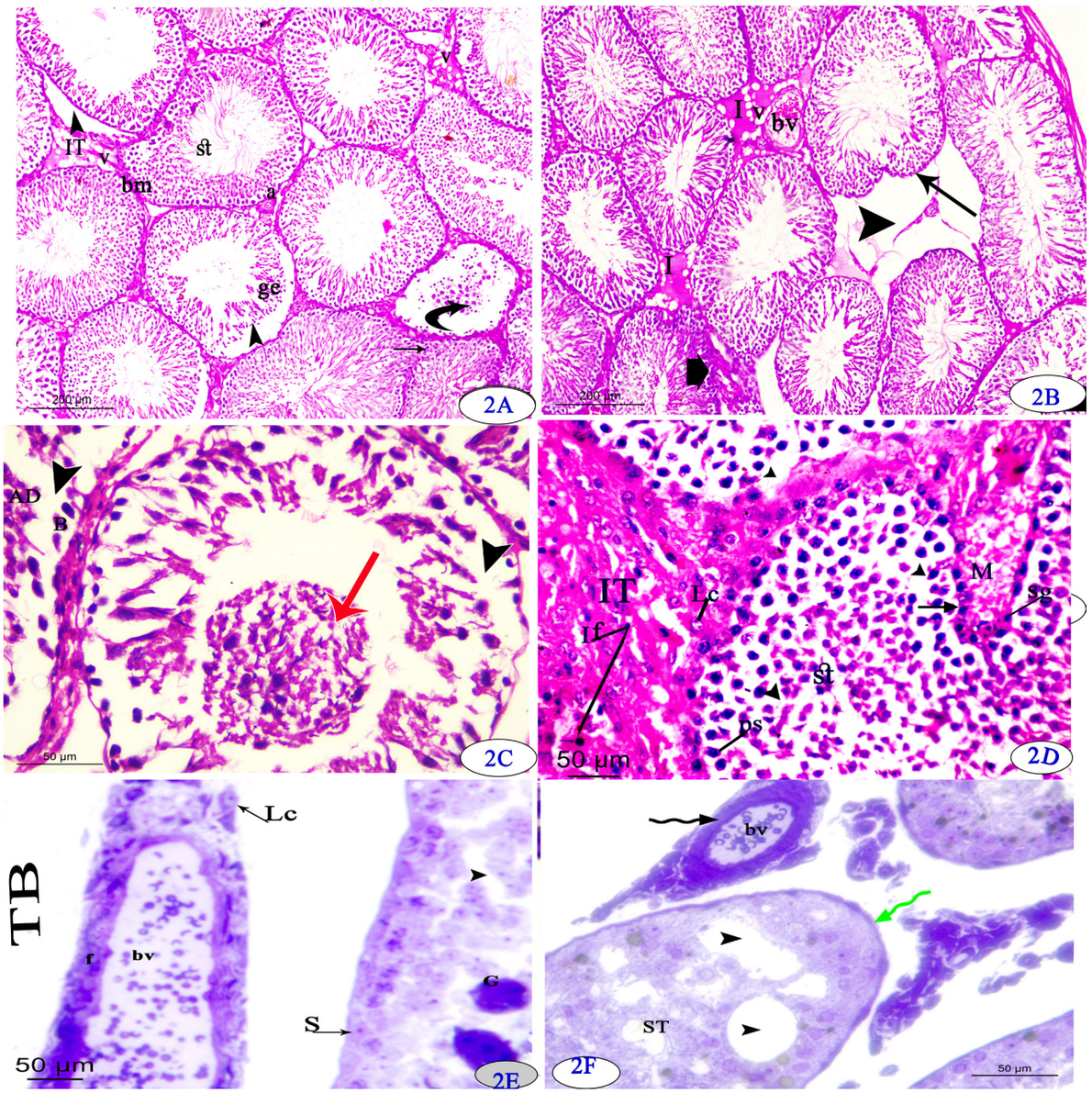

Fig. 2: Photomicrographs of sections in testicular tissue of diabetic rat (group III). [2A]: The seminiferous tubules (st) appear distorted and disorganized and ensheathed by basement membrane (bm). The germinal epithelium (ge) is separated leaving spaces (arrow heads) between its cells and the basement membrane (bm). The interstitial tissues (IT) are wide containing vacuolations(v) and blood vessel (a) with thickened wall. Notice, a degenerated tubule with desquamated cells in lumen (curved arrow), other tubule shows irregular and de-structed basement membrane (arrow). [2B]: shows the seminiferous tubules with empty spaces (arrow heads), in other areas, distortion of basement membrane (thick arrow), others with shrunken basement membrane (thin arrow). Wide interstitial space(I)was seen. containing many thickened walls blood vessels (bv) and oesinophilic substance and many vacuoles(V). (H&E x100). [2C&2D]: magnification of the previous figures [2C]: showing seminiferous tubule with disorganised germinal epithelium, wide intercellular spaces and desquamated epithelium (red arrow) in the lumen.Separation (arrowhead) of basal (b) and adluminal (Ad) cellular compartments in some ST. (Johnsen-like score 6). [2D]: this figure shows wide interstitial space (IT)and markedly increase in interstitial tissue (M)which encroaches and distorts adjacent basement membrane(arrow). Interstitial space contains numerous small darkly stained inflammatory cells (If) and Leydig cells (Lc). Few spermatogonia (sg) and spermatocytes (ps) with dark stained nuclei are present. [2E&2F] sections stained with toluidine blue[2E] shows Sertoli cell(S) rests on basement membrane ,germinal epithelium in adluminal compartment are disorganized showing wide intercellular spaces (arrow head) ,giant cells (G) are large dark cells with multiple nuclei ,blood vessel (bv) has thick wall and surrounded by dark stained elongated cells (f).Notice: Leydig cell (LC) is present .[2F]: shows seminiferous tubules (ST) with marked loss of architecture , empty spaces (arrow heads) and thickened basement membrane (green zigzag arrow) ,blood vessel (bv) has thick wall (black zigzag arrow) (A-B :Hx&Ex100, C-D Hx&Ex400 , E-F :toluidine blue x400).

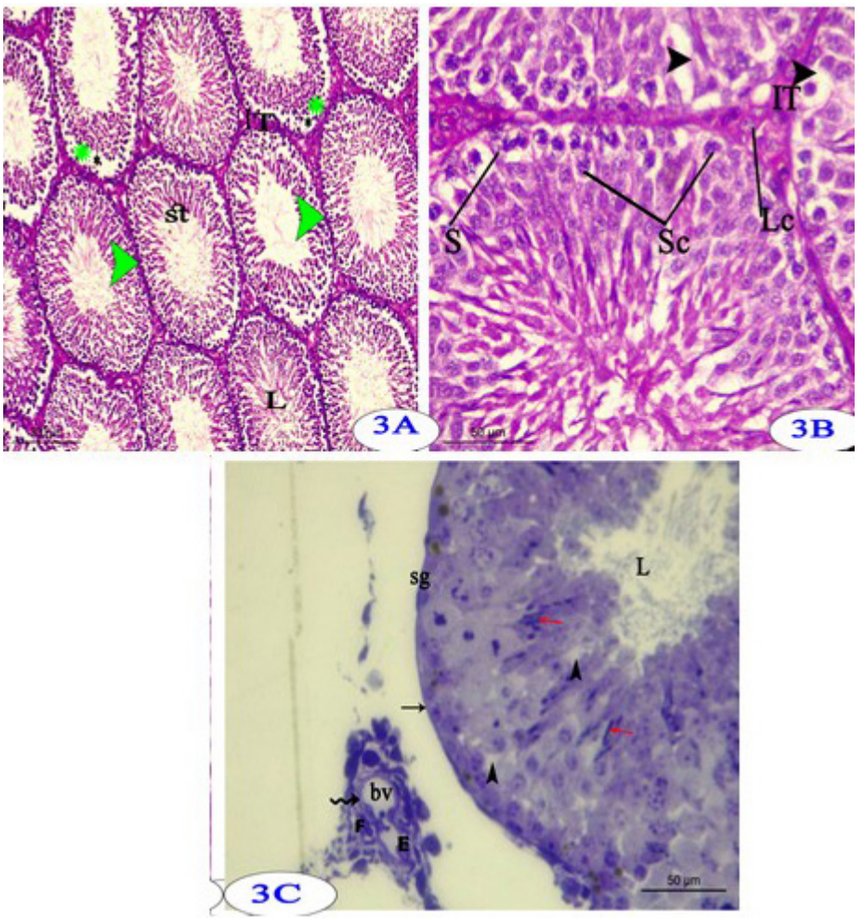

Fig. 3: Photomicrographs of sections in testicular tissue of Diabetic and vitamins group (group IV). [3A]: shows low magnification section of testicular tissue showing better organised seminiferous tubules(st) have regular basement membrane (green arrow heads) and lumina(L)filled with spermatozoa tails, few tubules have separation of adluminal part of germinal epithelium (green asterisks) from basal part. Notice: narrower interstitial space (IT) in-between tubules (Hx&Ex100). [3B]: higher magnification of the previous section shows seminiferous tubules with good organized layers of germinal epithelium ,Sertoli cell(S) rests on basement membrane ,many spermatocytes (Sc) are present .Some areas show wide intercellular spaces (arrow heads) .Interstitial tissue(IT)shows few Leydig cells(Lc) with vesicular nuclei .(Johnsen-like score ,9).(Hx&Ex400) .[3C]:section of testicular tissue stained by toluidine blue shows seminiferous tubule surrounded by regular and thin basement membrane (arrow) ,spermatogonia(sg)with dark nuclei rest on basement membrane , elongated spermatids (red arrows) embedded in between cells and lumen(L) is filled with sperm tails ,normally packed cells (arrow heads) .A blood vessel (bv) has thin wall(zigzag arrow) showing intact endothelial wall(E) ,and is surrounded by very few fibroblasts(f) .(toluidine blue x 400) .

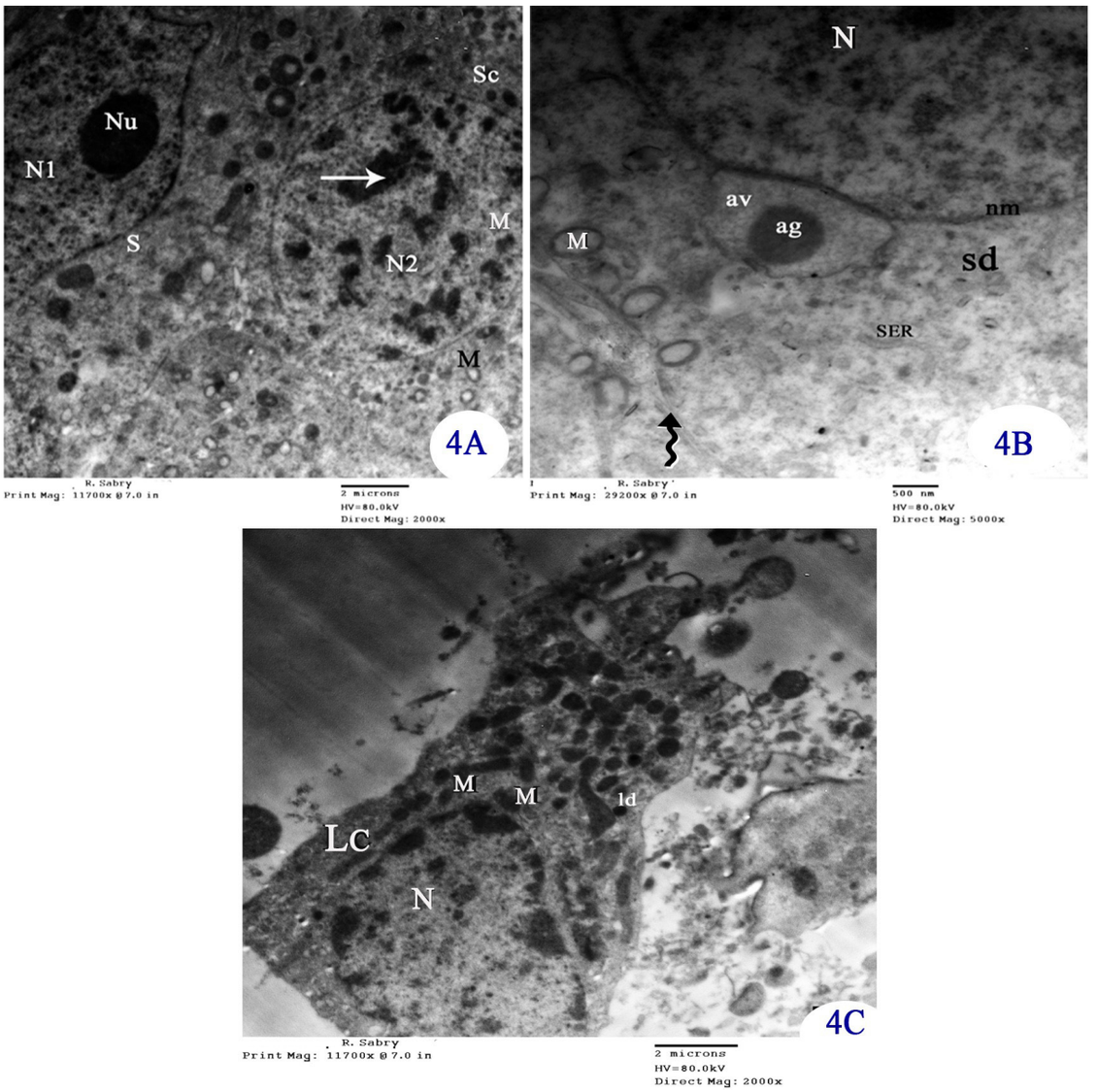

Fig. 4: Electron micrographs of seminiferous tubule epithelium of control group (groups I&II). [4A]: showing Part of Sertoli cell (S) has large euchromatic nucleus (N1) with prominent nu-cleolus (Nu), its cytoplasm contains many mitochondria. Adjacent spermatocyte (Sc) has large rounded euchromatic nucleus(N2) with heterochromatin chromatin clumps (arrow), its cytoplasm contains many mitochondria(M). [4B]: showing rounded spermatid (sd) has euchromatic nucleus (N) surrounded by distinct nuclear membrane (nm). An acrosomal granule (ag) within an acrosomal vesicle (av). Its cytoplasm contains vacuolated mitochondria (M) aggregated at the periphery of the cell and smooth endoplasmic reticulum (SER). Notice the tight junctions between spermatids (black zigzag zrrow). [4C]: showing Leydig cell (LC) has oval euchromatic nucleus (N) with marginated heterochromatin. Its cyto-plasm contains numerous electron dense mitochondria (M), lipid droplet (ld). [TEM [4A&4C] x2000, [4B] x5000.).

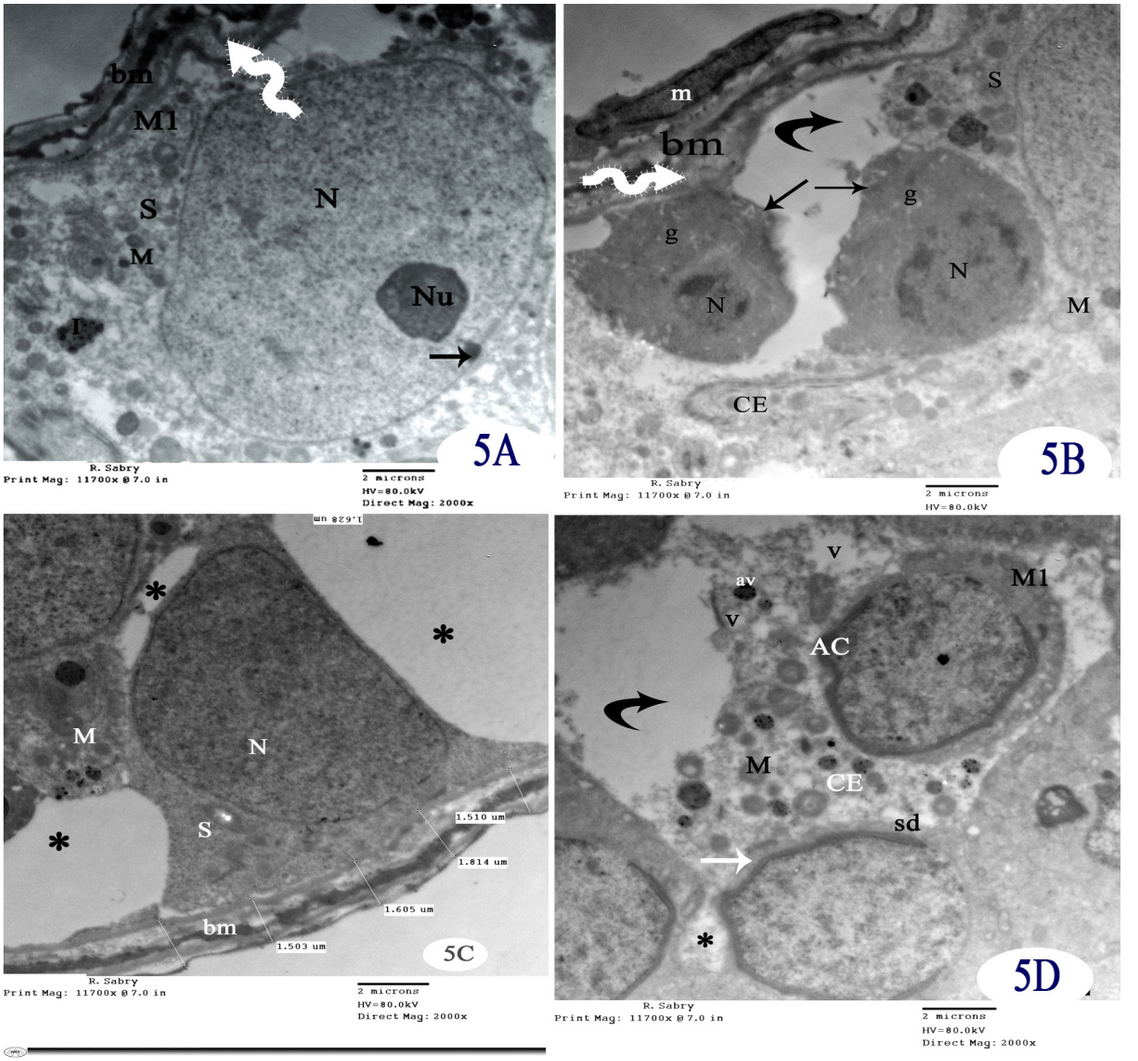

Fig. 5: Electron micrographs of seminiferous tubule epithelium of diabetic group (group III). [5A]: showing Sertoli cell(S) has large euchromatic nucleus (N) with peripheral clumps of heterochromatin (arrow) and prominent nucleolus (Nu), the cytoplasm contains many mitochondria (M) and inclusion bodies (I). Sertoli cell is widely separated from basement membrane (bm) which is thick and wavy with increased collagen deposition(white zigzag arrow)..[5B]: showing spermatogonia (g) appearing as ghost cells, resting on thick irregular basement membrane(bm) with increased collagen deposition (white zigzag arrow) and surrounded by myoid cell (m) .Spermatogonia(g) have irregular shrunken dark nuclei (N) , their cytoplasm is electron dense and surrounded by irregular cell membrane) arrows) .Wide spaces apparent between cells (curved arrows) , cytoplasmic extension (CE) of Sertoli cell (S) is seen containing many mitochondria (M) . [5C]: showing Sertoli cell(S) with triangular nucleus (N) surrounded by electron dense cytoplasm containing few mitochondria. wide intracellular and intercellular spaces (*) are present. Sertoli cell rests on thickened basement membrane (bm) .the figure shows measures of basement membrane thickness .[5D] : Multiple round spermatids (sd) have acrosomal cap (AC) spreading over the round nucleus ,cytoplasm contains many vesicular mitochondria (M1) interrupted intercellular junctions(*) and wide intercellular spaces(curved arrow) are noticed .Cytoplasmic extensions of Sertoli cells(CE) is present in-between cells ,it contains many vacuoles(V), dilated mitochondria (M) and cytoplasmic autophagic vacuole (av). (TEMx2000)

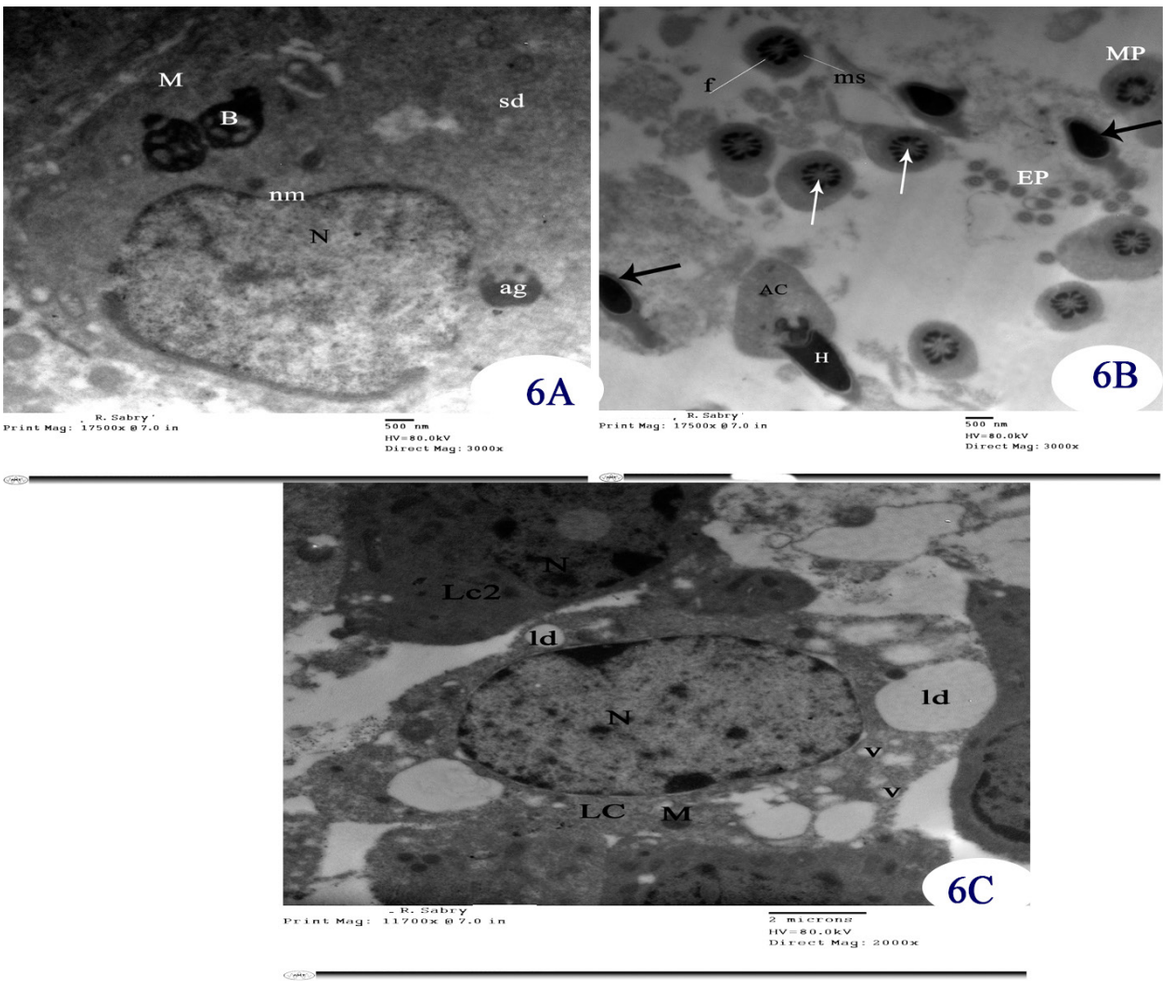

Fig. 6: Electron micrographs of seminiferous tubule of diabetic group (group III). [6A]: showing a spermatid (sd) has nucleus (N) with distorted nuclear membrane (nm). A detached acrosomal granule (ag), few mitochondria (M) and chromatin bodies (B) (x3000). [6B]: showing cross sections of end pieces (white arrows) and the middle pieces of spermatozoa tails have distorted electron dense fibers (f) with absent axonemes (white arrows), swollen mitochondrial sheath (ms). Longitudinal sections showing head (H), acrosomal cap (AC), other sperms lack acrosomal cap (black arrows). Multiple rounded end pieces (EP) are present. [6C]: showing Leydig cells (LC) with euchromatic nucleus (N) and clumps of heterochromatin. The cytoplasm contains few mitochondria (M), vacuolations (v) and lipid droplets (ld). Another degenerated Leydig cell (Lc 2) has dark apoptotic nucleus (N) with ill-defined nuclear membrane, cytoplasm is dark with ill-defined organelles (TEMx2000, scale bar : 2 um).

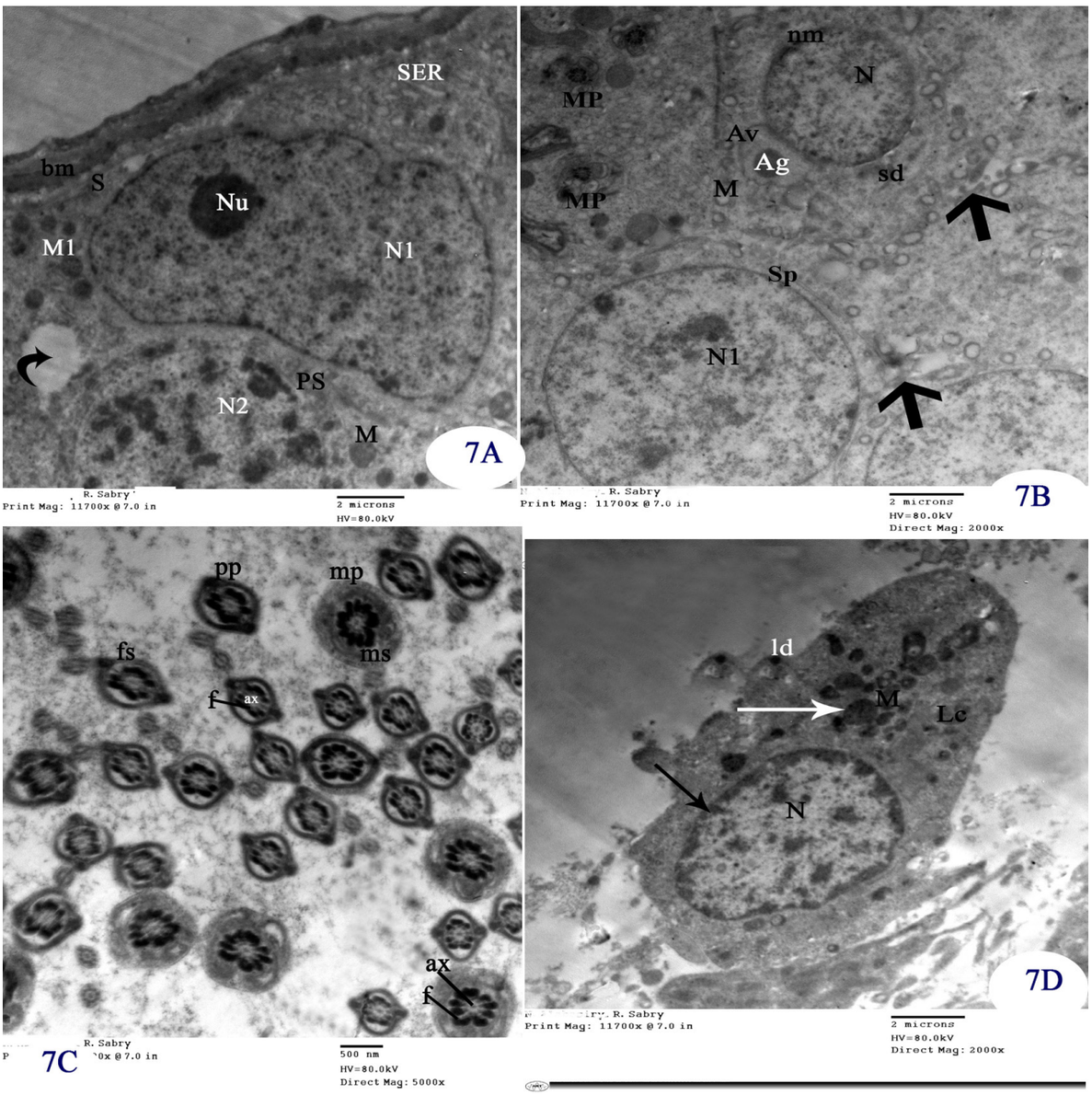

Fig. 7: Electron micrograph of seminiferous tubule of C& E treated rat testis (group IV),[7A] : showing Sertoli cell (S) resting on thin regular basement membrane (bm). It has euchromatic indented nucleus(N1) with prominent nucleolus (Nu), its cytoplasm contains many mitochondria (M) and smooth endoplasmic reticulum (SER). an adjacent primary spermatocyte (PS) has large rounded euchromatic nucleus(N2) with dispersed dense irregular clumps of heterochromatin, its cytoplasm contains rounded mitochondria(M1) Notice: small intercellular space (curved arrow) is also seen. Better intercellular relations than diabetic group with no wide intercellular spaces are seen. [7B]: showing round spermatids (sd) in Golgi phase of spermiogenesis , rounded euchromatic nucleus (N) surrounded by distinct nuclear membrane (nm) , An acrosomal vesicle (AV) containing acrosomal granule (Ag), cytoplasm has many mitochondria (M) at the periphery, a spermatocyte (Sp/) has large euchromatic nucleus (N1) , sections of middle piece of spermatozoa (MP),Notice: intercellular junctions between germ cells are seen(arrows).[7C] : showing cross sections of spermatozoa tails . The middle pieces (mp) section shows intact central core of axoneme (ax) surrounded by nine electron dense fibers (f) and outer electron dense mitochondrial sheath (ms). The principal pieces shows intact core of axoneme (ax) surrounded by nine dense fibers (f) and outer fibrous sheath (fs) .[7D]: showing Leydig cells (LC) with oval euchromatic nucleus (N) with marginated heterochromatin (arrow). The cytoplasm is electron dense and contains numerous electron dense mitochondria (M), few swollen mitochondria (white arrow) and lipid droplets (ld). (7C:x5000, scale bar:500nm,7A,7B and 7D x2000, scale bar :2um)

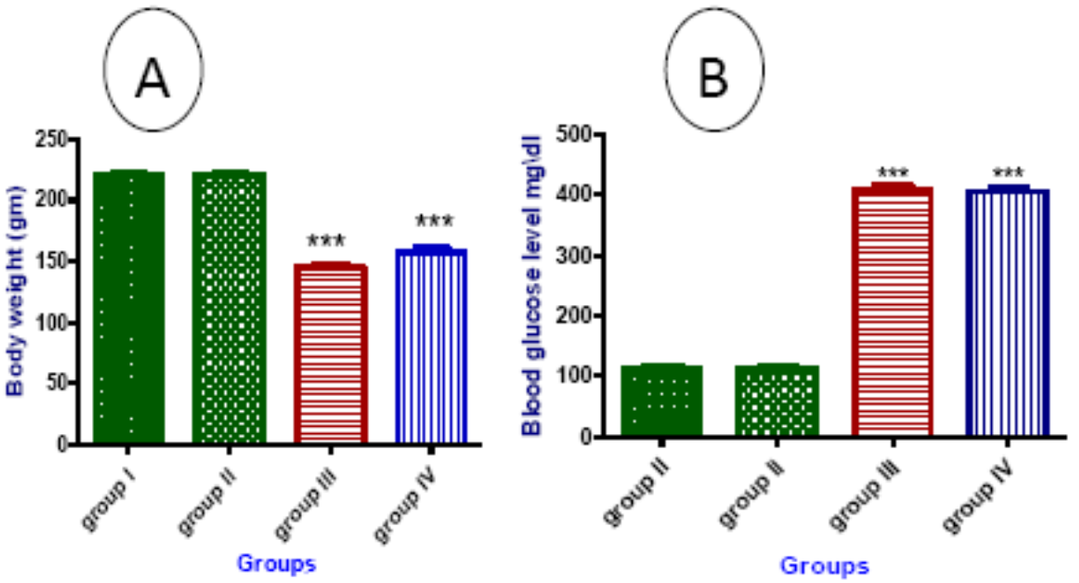

Fig. 8: A: Body weight (gm) among groups, *** highly significant difference between control and treated and between group III& group IV B: Blood glucose level (mg/dl) among groups, *** highly significant difference between control and treated.

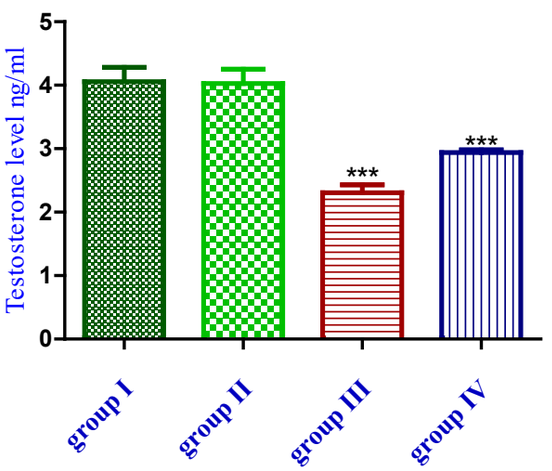

Fig. 9: Bar Chart showing blood testosterone level ng/ml among groups, *** highly significant difference between control and treated (*P value* < 0.0001)

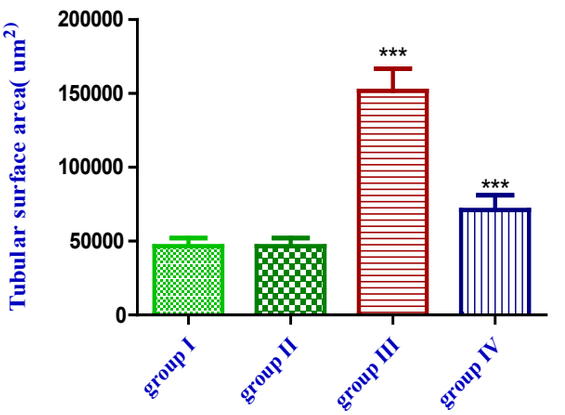

Fig. 10: Bar Chart showing tubular surface area (µm2) among groups, *** highly significant difference between control and treated and between group III & group IV (*P value* < 0.0001)

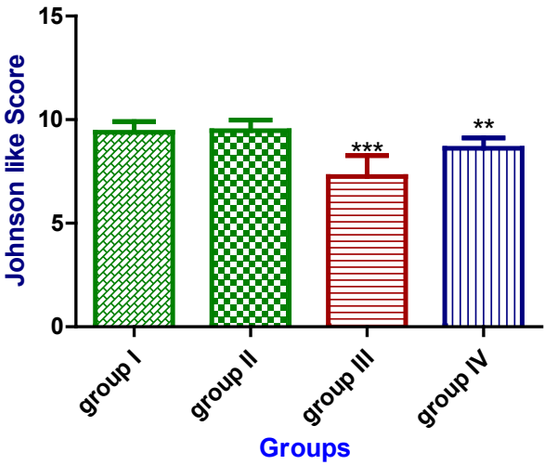

Fig. 11: Bar Chart showing Johnson like score among groups, *** highly significant difference between control and treated (*P value* < 0. 0001) and **significant difference between group III & group IV (*P value* < 0. 001).

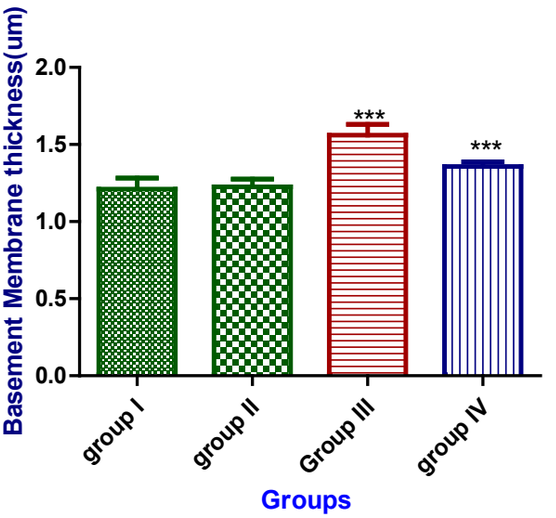

Fig. 12: Basement membrane thickness (µm) among groups, *** highly significant difference between control and treated and between group III & group IV (*P value* < 0.0001) .

Table 2: Means and standard deviation of body weight and blood glucose level in different groups

parameter	group I	group II	group III	group IV
Body weight (g)	219.7±2.8^{bc}	220±3.0^{bc}	145.1±2.1^{a}	158.8±5.2^{a}
Blood glucose level (mg/dl)	113,2±8.1^{bc}	112.9±7.8^{bc}	407.6±22.5^{a}	405.2±20.1^{a}

Statistically significant at $P \leq .05$ (a: statistically significant with controls *P value*≤ .001***, b: statistically significant with the diabetic rats group *P value* ≤.001*** , c: statistically significant with the vitamin C&E-treated diabetic rats $P \leq .01$**)

Table 3: Means and standard deviation of serum testosterone, tubular sur-face area, Johnsen like score and basement membrane thickness in different groups

parameter	group I	group II	group III	group IV
Testosterone(ngLml)	4.05±0.74^{bc}	4.02±0.75^{bc}	2.3±0.41^{a}	2.9±0.14^{a}
Tubular surface area (um^2)	46635±5417^{bc}	46631±5418^{bc}	151689±14981^{ac}	71165±9853^{ab}
Johnsen like score	9.4 ±0.51^{bc}	9.467±0.52^{bc}	7.25 ±1.02^{ac}	8.62±0.5^{ab}
Basement Membrane thickness	1.21 ±0.07^{bc}	1.23±0.05^{bc}	1.56 ±0.07^{ac}	1.36±0.03^{ab}

Statistically significant at $P \leq .05$ (a: statistically significant with controls *P value*≤ .001***, b: statistically significant with the diabetic rats group *P value* ≤.001*** , c: statistically significant with the vitamin C&E-treated diabetic rats $P \leq .01$**)

DISCUSSION

Diabetes Mellitus is a metabolic endocrine disease that yields to many disorders in various body organs. By its increasing prevalence, it will be considered a major noncommunicable disease in about 20 years[28]. This study investigated whether prior treatment with combination of vitamins C&E could be protective against diabetesinduced testicular injury. In this study induction of diabetes was produced by streptozotocin as streptozotocin induced diabetes gives a relevant model to evaluate diabetic effects on various organs. Its effects on male reproductive tissues are similar to those detected in human[29].

In the present study, combination of vitamins C& E was chosen, as vitamin C acts as an antioxidant in conjugation with vitamin E. Vitamin C is important in vitamin E reformation; it helps in reduction of electron tocopherol radical to tocopherol. So, these coadministration of the two vitamins produce a synergistic action and reinforces its antioxidant potential[29]. Moreover, in the present study we use Vitamin E in the form of tocopherol and this in line with[30] who stated that Vitamin E is an antioxidant that includes many subtypes. Among them, atocopherol is the most one that has antioxidant activity.

According to body weight there was significant weight loss in both diabetic and diabetic and vitamins group in comparison with control groups which was in consistence with El Kotb *et al*.[31]. Many structural and functional changes in various organs were produced by elevated blood glucose level in diabetic patients[32]

In the present work, the blood glucose measures of diabetic rats were elevated above the normal level and this increase was approximately constant throughout the course of the experiment. While there was nonsignificant difference with vitamins supplementation. On contrast. Alasmari *et al*.[33] demonstrated significant decrease in blood glucose level in vitamin E treated group.

The elevated blood glucose level enhances free radicals production and diminish antioxidant capability so increasing oxidative stress in testis[34,35]. These findings can explain the results in the present study, despite VCE treatment did not significantly decrease the blood glucose level, however there was improvement of histopathological and ultrastructural changes of diabetic testicular tissue via their antioxidant effects. These findings can be explained by the study of Kianifard[36] who reported that increased oxidative stress and impaired energy metabolism occurs in diabetes. It is suggested by experimental studies that oxidative stress and producing reactive oxygen species (ROS) are the main insult in the pathogenesis of both types of diabetes mellitus. Vitamin E has antioxidant role as it diminishes oxygen free radicals formation[37].

Concerning the present study, examination of the diabetic rats testis by light microscopy showed that the seminiferous tubules of diabetic group had irregular outline, degeneration of germinal epithelium and Sertoli cells, many spaces devoid of testicular tissue in seminiferous tubules, decreasing number of germ cells and Sertoli cells. So, spermatid production was reduced in most tubules, few sperms and empty wide lumina of tubules were encountered as previously evidenced by many studies[38,39]. These diabetic histopathologic changes were ameliorated by vit C&E supplementation, and this was in accordance with Ayoubi *et al*.[40] who found that based on histological findings, Diabetes affected the process of spermatogenesis. Germinal layers thickness was diminished in diabetic; however this effect was improved by vitamin C.

The wide interstitial spaces and increased acidophilic material were seen in diabetic group in the current study. Other studies reported the same findings[33,41]. The interstitial acidophilic material may be due to lymphatic exudates. Also, increased permeability of congested veins can contribute[42].

Oxidative stress was believed to be the main cause in pathogenesis of diabetes mellitus induced male reproductive defects[43]. In diabetes, spermatogenesis was mainly impaired by mitochondrial dysfunction. The latter was a result of increased mitochondrial reactive oxygen species which were generated by accelerated cellular respiration[44,45].

While treatment with vitamins in diabetic rats significantly increased spermatogenesis and germ cell count, increases in all spermatogenic cells, leading to increased spermatids and spermatozoa when compared to untreated diabetic group. Vitamins could prevent the increased formation of free radicals, produced by spermatozoa, by means of their antioxidant property[16]. Also, Ayeleso *et al*.[46] reported that antioxidant vitamins such as vitamin A, C and E are part of the biological nonenzymatic protection from oxidative stress.

The histological, ultrastructural, and morphometric results of diabetic group in this study showed thick and irregular electron dense basement membrane with deposition of collagen fibers surrounding some seminiferous tubules, others showed irregular and disrupted basement membrane. This means that the basement membrane had altered structure, and this could severely impair testicular function. These results were in consistence with previous literatures[47,48,49] that referred these pathological effects on testicular tissue to an increased thickness of basement membrane.

The present study noticed that treatment by vitamins in diabetic rats preserved the structure and integrity of basement membrane in most seminiferous tubules, and significantly decreased the thickness of basement membrane. This was in accordance with the study of Omar *et al*.[19], who observed a decrease in thickness of seminiferous tubules basement membrane in diabetic rats on using vitamin E supplementation, as well as with Alasmari *et al*.[33], who demonstrated an increase in the deposition of collagen fibers around seminiferous tubules of diabetic groups, while it decreased in vitamin E treated group.

In the present study the Sertoli cells showed apoptosis, degeneration and cytoplasmic vacuolations which were more in basal than in apical part. Many previous studies confirmed Sertoli cell injury in diabetic rats[50,51].

Multivesicular giant cells were present in the testicular tissue in diabetic group. Giant cells were considered a pathognomonic sign of atrophy of testis[52]. It is produced by amalgamated spermatids due to alterations in the intercellular bridges, impairment of cytokinesis[53] or phagocytosis of degenerated spermatogenic cells[54].

Wide separation and impaired close relationship between germ cells and Sertoli cells were noticed in diabetic group. However, restoration of the close relationship between cells occurred in vitamins treated group which improved spermatogenesis as demonstrated by Maresch *et al*.[55], who reported an improvement of the bloodtestis barrier and many metabolic properties of testicular cells that regulate the blood to germ cell glucose and metabolites transmission.

Moreover, in the current study, Sertoli cell structure was preserved with obvious decreased cytoplasmic vacuolations in vitamins treated group, these results were in consistence with previous works[56,57]. In *vitro* studies had demonstrated that the extracellular matrix plays a crucial role in Sertoli cell function[58].

Concerning Leydig cells, in the current study, abnormal distribution was detected in diabetic rats. This was described by Cao *et al*.[59] who recorded highly decreased number

of Leydig cells in some areas of rat testis. The observed Leydig cell hyperplasia was proposed to be a compensatory mechanism for the lowered serum testosterone level reported in the present study or related to augmented activity of Leydig cells as previously reported[60]. Diabetes appeared to have the same effects as aging, it elevated the oxidative stress in the testicular tissue where antioxidant enzymes were abundant and vital for the preservation of testicular function[61]. Another study reported a decrease in Leydig cells, as diabetes caused oxidative stress stimulating inflammatory cells, as macrophages, to secrete cytokines. This might lead to inhibition of Leydig cells and impaired spermatogenesis, which was in line with the appearance of inflammatory cells in other areas in the present study[62]. These findings also explained normal distribution of Leydig cells in diabetic rat treated by vit C&E in present study.

In the current study, the blood tes-tosterone level in diabetic rats was diminished. Diabetes can produce a reduction in the biosynthesis and metabolism of androgens, this has been reported in many studies[63,64]. While other studies reported a decrease in the blood testosterone level in diabetic rats, but this decrement was not sig-nificant[65,66]. These discrepancies may be related to different strains of rats and/or methods of studies. The mechanism of reduction in blood testosterone level might be a direct effect of elevated blood glucose level or its metabolites on gonadotropins or due to cellular resistance to these hormones[67]. Studies on diabetes reported a reduction in blood plasma LH levels and decreased number of LH binding sites in Leydig cells which is responsible for normal function of these cells[68]. Consequently, there was a reduction in testosterone level inspite of increased Leydig cells in some areas in the current study. These processes led to depression of synthesis and secretion of testosterone by Leydig cells[69].

In the present study, there was mild statistically nonsignificant increase in blood testosterone group in vitamins treated diabetic group in comparison with diabetic group which is on contrary to the study of Alasmari *et al*.[33] who found significant increase in vitamin E treated group. This difference may be due to longer duration of experiment in their study (8 weeks). The mild increase in testosterone level in diabetes and vitamins group despite of observed hyperglycemia can be explained by Diabetes related hyperglycemia that induced the production of free radicals and destroyed the endogenous antioxidant defense systems by different mechanisms which could be ameliorated by antioxidants[70,71]. In the current study there was no significant decrease of hyperglycemia in diabetes and vitamins group and this finding is on con-trary to the study of Gamal *et al*.[72] who demonstrated that vitamin C administration could decrease the high levels of blood glucose, cholesterol, triglycerides, and LDL in diabetic group.

In the present study, there was an increase in tubular surface area in diabetic group due to intercellular dilated spaces. This finding was in consistency with Malarvani *et al*.[73] who reported degeneration of cells and edematous hypertrophied seminiferous tubules in diabetic rats. This oedema was mainly due to dilated, congested blood vessels and endothelial dysfunction in diabetes.

Shi and Vanhoute[74] postulated that in diabetes there was endothelial dysfunction in macro and microvascular channels. This abnormal endothelial function, as a microvascular complication, was concomitant with decreased release of nitric oxide, elevated oxidative stress, and accumulation of inflammatory products. This finding explained the decrease in vascular edema in tubules in vitamin treated group in the present study. Another important factor contributing to oedema was the impairment of integrity in basement and cellular membranes in diabetic rats. The basement membrane structure was thought to regulate fluid transmission from the intertubular medium toward the seminiferous tubules lumina[75].

Increased levels of free radicals in diabetes could produce damage of cellular organelles and high lipid peroxidation in the plasma membranes[76,77]. These results were in contrast to other studies[34,78] that found a decrease in tubular diameter in diabetes. This discrepancy may be due to different duration of experiment. As early in diabetes, there was interstitial and intercellular oedema. Acute stress can prompt intercellular space dilatation[79]. While with progression of the disease, fibrosis and atrophy of tubules progress with time so in the present study we found some blood vessels surrounded by active fibroblasts. This was supported by Young and her colleagues who found that active fibroblasts called myofibroblasts are present during repair of degenerated tissue producing early fibrosis[80]. This beginning of fibrosis, in the form of aggregation of fibroblasts around blood vessels, was encountered in some areas of testicular tissue in the current work.

In the present study, there was thickening of some blood vessels in diabetes while this finding was ameliorated by treatment with vitamins C&E which can be explained by Rask Madsen and King[81] who said that main cause of diabetic vascular problems is the imbalance between molecular causes of injury and endogenous defense factors as anti inflammatory factors and antioxidant enzymes. The important histopathologic changes in blood vessels in diabetes are endothelial cell apoptosis, increased permeability and thickening of basement membrane.

In diabetes there was increased phosphorylation of occluding element of tight junctions producing vascular leakage[82]. Long and his colleagues found thickening of blood vessels in diabetic rats while thinner wall of blood vessels was noticed in rats treated by antioxidants[83].

Moreover, Maha *et al*.[84] demonstrated that vitamin E and vitamin C could decrease macro vascular complications of diabetes by protection against free radicals and lipid peroxidation.

Some tubules surrounded by thick basement membrane with deposition of collagen fibers and few atrophied shrunken tubules. This tubular oedema was the cause of significantly

increase in tubular surface area in diabetic group in spite of decreased Jonhsen like score. In the present study, the treatment with vitamins early in the disease improves vascular and cell membrane integrity, decreases oedema and so delays progression of the disease toward fibrosis and tubular atrophy. Consequently, this improvement leads to significant improvement in spermatogenesis which is encountered by increased Johnsen like score. This finding was in line with Mruk and Cheng[85] who reported that the metabolism of glucose and lactate formation (the preferred energy source for germ cells) take place in the cytoplasm of Sertoli cells. Accordingly, the diabetic hyperglycemic microvascular changes of testicular tissue can affect the transport of glucose and hence lead to altering structural and functional spermatogenesis due to change of cellular nutrition. Induction of diabetes is associated with cellular modifications in testicular micro-environment[86].

In the current study there was statistically significant decrease in the mean Johnsen like scores in comparison to vitamins treated group indicating significant improvement of spermatogenesis by vitamins coadministration and this was in contrary to another study[87] that reported that vit E produce no significant result with regard the seminiferous epithelial germinal thickness. This result could suppose that combination of vitamins has better results. However, the increase in germ cell count in the present study is in line with Asmat *et al*.[88] who demonstrated that Vitamin E can increase sperm number.

CONCLUSION

In conclusion, the treatment with combined vitamin C plus vitamin E in dia-betes was effective in reducing oxidative stress and oedema of testes and significant-ly increased Johnsen like score. Therefore, Vitamins having antioxidant properties should be very important in nutrition of healthy individuals to protect them against pathological hazards of diseases. Also in routine clinical treatment these antioxidants may be used as supporting therapy for re-productive dysfunction.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الدور الوقائي المحتمل للغذاء المكمَّل بخليط من فيتاميني ج و هـ على خصية الجرذ الابيض البالغ، مستحث الاصابة بداء السكري: دراسة للبنية الدقيقة والقياسات الشكلية والبيوكيميائية

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الخلفية: الإجهاد التأكسدي له دور في تسبب مرض السكري بإصابة الخصية مما يؤدي إلى تطور وتضخم اضطرابات الخصية نتيجة مرض السكري.

الهدف من هذه الدراسة: هدفت هذه الدراسة إلى تقييم التأثيرات التحسينية للعلاج بالفيتامينات ج وهـ مرة قبل ومرة مصاحبا لمرض السكري على التغيرات النسيجية المرضية والتغيرات الهيكلية والكيميائية الحيوية والتغيرات الشكلية الناتجة عن مرض السكري.

المواد والطرق المستخدمة: تم استخدام أربعين جرذ بالغ أمهق في هذه الدراسة. وتم تقسيمهم إلى أربع مجموعات متساوية: المجموعة الضابطة (المجموعة الأولى): تتناول الطعام والماء بحسب الرغبة)؛ المجموعة الضابطة الموجبة (المجموعة الثانية): يُعطى الحقن داخل الصفاق من محلول السترات فقط ٠,١ مل ، ودرجة الحموضة = ٤,٥) ؛ مجموعة مرضى السكري (المجموعة الثالثة): ٤٠ مجم / كجم من الستربتوزوتوسين المذاب في محلول السترات داخل الصفاق ومجموعة مرضى السكري مع فيتامين ج و ه (المجموعة الرابعة أو مجموعة فيتامينات ج و ه): نظام غذائي مكمل بفيتامين ج و ه لمدة ستة أسابيع (أسبوعين قبل إحداث مرض السكري وأربعة أسابيع بعد الإحداث). تم قياس مستوى هرمون التستوستيرون في الدم، ورفع قياسات شكلية لمساحة الأنابيب المنوية، وتحليل صورة سمك الغشاء القاعدي.

النتائج: بالتركيب الدقيق، في مجموعة مرض السكري، كان هناك غشاء قاعدي سميك ومشوه ومسافات واسعة بين الخلايا. من الناحية الشكلية، أدى مرض السكري إلى زيادة مساحة السطح الأنبوبي وتم تحسين هذه التغيرات مع العلاج بالفيتامينات السابقة حيث كان الغشاء القاعدي رقيقًا ومنتظمًا وأيضا زيادة في الطبقات الجرثومية. على مستوى البنية الدقيقة في مجموعة مرضى السكري وجد أنه زادت سماكة الغشاء القاعدي وتشوه شكله واتسعت المسافات البين خلوية. على مستوى القياسات الشكلية فإن داء السكري ادى لزيادة مساحة سطح الانابيب. هذا وقد ادى تناول المسبق لخليط الفيتامينات الى التقليل من هذه التغيرات حيث وجد ان الغشاء القاعدي حافظ على انتظام شكله وقلة سمكه بالإضافة الى زيادة الطبقات الجنينية/ الجرثومية. الاستنتاج: يستحسن ان تضمن الفيتامينات ذات الصفات المضادة للأكسدة في النظام الغذائي للأفراد الصحيحة وان تستخدم كعلاج مساعد في حالات داء السكري لتساهم في تقليل المخاطر المرضية لهذا الداء.

الخلاصه: يجب أن تكون الفيتامينات ذات الخصائص المضادة للأكسدة مهمة في تغذية الأفراد الأصحاء وكعلاج مصاحب لمرض السكري لتخفيف المخاطر والتأثيرات الضارة علي الأنسجة.