Original Article

Comparative Histological and Immunohistochemical Study on the Effects of Antidiabetic Drugs (Metformin Versus Sitagliptin) on the Testes of Adult Male Albino Rat

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ABSTRACT

Background: Many complications have been detected for diabetes mellitus which is considered one of the most dangerous metabolic diseases. Little is known for reproductive complications of oral antidiabetic drugs.

Aim of Work: This study aims to evaluate and compare the effects of common oral antidiabetic drugs (Metformin and Sitagliptin) on adult male albino rat testes through biochemical, histological, immune histochemical and morphometric studies.

Material and Methods: Fifty adult male albino rats were used. They were divided into three groups, control group (GI), Metformin group (GII) and Sitagliptin group (GIII). Testes were cut off and processed for histological study by using Hematoxylin and Eosin (H&E), Mallory trichrome (M.T) and electron microscope examination. Immunohistochemical examination as Proliferating cell nuclear antigen (PCNA), Androgen receptor (AR), and Caspase3 stains were done. Serum testosterone, Malondialdehyde (MDA), sperm count and motility were measured. Morphometric studies were also done.

Results: Testes of Sitagliptin rats show marked destruction of many seminiferous tubules (S.Ts). Damage of their lining germinal epithelium and vacuolation of its cytoplasm and marked reduction in number of sperms in lumen are seen. Interstitial Leydig cell show degenerative changes. Marked deposition of collagen fibers between S.Ts is observed. Strong positive reaction for Caspase 3 and weak reaction in PCNA and AR stains are seen.

Testes of Metformin rats show less histopathological changes. Many tubules have nearly intact layers of germinal epithelium, and some sperms in lumen. Moderate deposition of collagen fibers between tubules is noticed. Moderate reaction with Caspase 3, PCNA and AR stains are observed. These changes are in harmony with electron microscopic findings which are seen in all groups.

Conclusion: Both Metformin and Sitagliptin drugs have destructive histological effects on the testes but Sitagliptin has more marked effect. So, in young aged diabetic male, it's preferable to use Metformin drug with continuous follow up.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder disease which is distinguished basically by hyperglycemia and it leads to many complications in various internal body organs including genital system[1] Fertility rates in modern communities have revealed that, increased incidence of infertility associated with DM in both men and women[2].

Infertility associated with DM can be referred to sex steroids hormonal deregulation[3]. Metformin (dimethyl biguanide) is a drug that decreases glucose level of blood and enhances the glucose tolerance without changing the profile of plasma insulin. It has been used clinically for more than 50 years especially in patients with type 2 diabetes as the first choice of treatment[4]. Although, Metformin (MET) is one of the most used antidiabetic drugs it is also considered as a potential cancers therapy[5].

Metformin (MET) improves catabolism of glucose by affecting metabolic pathways of insulin and increasing sensitization of cells to insulin. Also, Metformin enhances adenosine monophosphate-activated protein kinase (AMPK), which is a main factor for energy balance. Although MET is clinically useful, its tissue toxicity is not clearly studied[6].

Sitagliptin is a recent antidiabetic therapy, it suppresses the dipeptidyl peptidase-4 (DPP-4) enzyme and stimulates insulin liberation in blood. Furthermore, it enhances proliferation of beta-cell[7]. Continuous evaluation of side effects of Sitagliptin from clinical usage is recorded[8].

Few studies for antidiabetic drugs complications on reproductive systems have been estimated[9]. Thus,this study has been prepared to evaluate and compare

biochemical, histological, immunohistological and morphometric effects of antidiabetic drugs (Metformin and Sitagliptin) on testes of adult male albino rat.

MATERIALS AND METHODS

Materials

Animals

This research was done on 50 adult male albino rats weighing 190-200 g.

Animals were kept in clean ventilated rooms in the animal house of the Faculty of Medicine, Menoufia University, Shebinelkom, Menoufia, Egypt. Laboratory diet were used for feeding. Animal Care and Ethical Committee guidelines were applied on the rats.

Drugs

Sigma-Aldrich Corp. (USA) is our source where all drugs and chemicals were purchased from. Drugs were dissolved in distilled water and were used daily for 6 weeks by oral gavage. Rats were divided into three groups at random way: Group I (control) composed of 10 rats; that received distilled water vehicle. Group II (Metformin treated) composed of 20 rats, that received Metformin at the dose 100 mg/kg/day) according to Sun *et al.*[10]. Group III (Sitagliptin treated) composed of 20 rats, that received Sitagliptin (30 mg/kg/day) according to Chen *et al.*[11].

Methods

I-Biochemical study: Investigations were done at Biochemistry Department, Faculty of Medicine, Menoufia University.

• Testosterone hormone (TH)

At the end of the experiment, collecting blood samples in capillary tubes treated with heparin from tail vein were done. Commercial kits for measurement of serum levels of testosterone hormone (TH) by radioimmunoassay were used.

Tissue sampling

Anesthesia by ketamine (90 mg/kg) / xylazine (15 mg/kg) intraperitoneally[12] for all animal groups were done. Then animals were scarified, tissue were gotten out and perfused with 10% neutral-buffered formalin.

• Sperms count: Both right and left cauda epididymides were dissected. One cm incision was done to release all sperms in collecting vial containing 5ml bovine serum albumin (BSA)-Hanks solution. Then, filtering this fluid through a nylon mesh sieve and diluting with formalinized saline were done. Epididymal sperm counts was done by using the hemocytometer. Then multiplying sperm number by the diluting factor was performed to obtain the total sperm count[13].

• Progressive motility

The progressive motility of sperms was estimated according to the method reported by Bearden and Fuquay[14].

• malondialdehyde (MDA)

By using colorimetric assay kits, MDA level (lipid peroxidation parameter) was calculated. Specimens of testes were taken and prepared properly according to Ohkawa *et,al*[15].

II-Histological study: A) Light study: Testes were put in Bouin's solution and were prepared for paraffin blocks. 4 mm thickness sections were cut and stained with Hematoxylin and Eosin (H and E) and Mallory Trichome (MT)[16].

B) Ultrastructure Study: tissue samples from 5 rats in each group were processed. A small part was excised rapidly (within 1 min) and minced into 1x1 mm² piece. Then, proper fixation and staining with toluidine blue to make semithin sections (1 mm thick) were made and then were examined by light microscope. Finally, ultrathin sections were made and examined with the transmission electron microscope (Seo-Russia) in Tanta E.M Center at Faculty of Medicine Tanta University[17].

III- Immunohistochemical study: Staining was performed on 4-mm thick sections.

Nocturnal incubation of the tissue sections was done at room temperature, with the next primary antibodies:

A. Antiprimary antibodies to (PCNA) (Clone PC 10; Dako Denmark A/S, Glostrup, Denmark)[18],

B. Primary monoclonal and polyclonal antibodies for androgen receptors (Cat. No. MA1-150; Thermo Fisher Scientific Co., Waltham, Massachusetts, USA)[19].

C. Anti-caspase-3 [mouse monoclonal antibody (Lab Vision, USA)[20].

Controls slides were included in each run. Negative controls were made by primary antibody exclusion.

IV- Morphometric study: It included measurement of the following parameters:

A. Mean diagonal diameter of STs were measured in H&E-stained sections at nearly rounded position ($\times 100$).

B. Mean height of spermatogenic epithelium of seminiferous tubules (STs) were measured in H&E-stained sections. Five measurements were done to gain its average ($\times 400$).

C. Average number of Sertoli cells were counted. (x 400),

D. Average number of PCNA immunopositively cells, only the basal germ cells of the seminiferous tubules were counted as they were the cells in which active DNA synthesis had happened[21]. ($\times 200$)

E. Average number of AR immunopositive cells were counted.

F. Caspase 3 immunopositive cells (×200) were counted.

From various sections of each group, all measurements were made in 10 non overlapping areas. Image analysis was performed using Leica Qwin 500 LTD software image analysis computer system (Cambridge, England) at the Anatomy Department, Faculty of Medicine, Menoufia University.

Statistical analysis: It was done for all biochemicals and morphometrical results. Statistical analysis of data was made by SPSS, version16.00(Chicago, Illinois, USA).

Expressed data were done as mean ± SD and then were analyzed by using one-way analysis of variance test followed by post-hoc for comparison between groups. Significance was considered at $p<0.05$.

RESULTS

Biochemical results

Serum testosterone level

Serum testosterone hormone level is showing significant decrease in the MET group ($P<0.05$) and is showing highly significant decrease in Sitagliptin group ($P<0.001$) in comparison with the control group (Table 1 and Histogram 1).

Testicular oxidative stress markers (MDA)

The testicular level of MDA is showing significant increase in MET group ($P<0.05$) and is showing highly significant increase in Sitagliptin group ($P<0.00.1$) when compare with the control group (Table 1 and Histogram 2).

Epididymal spermatozoa examination

Metformin group show a significant decrease ($P<0.05$) in the sperm count and non-significant relation ($p>0.05$) in motility when compare with the control group . Sitagliptin group show a high significant decrease ($P<0.001$) in the mean sperm count and motility when compare with the control groups (Table 1 and Histograms 3 & 4).

Histological study

Light microscope examination

The testes of the GI (control group) stained by H&E show that testicular parenchyma consists of densely packed Seminiferous tubules (STs) with little interstitium (Figure 1). The STs are surrounded by regular thin basement membrane and are lined with stratified germinal epithelium which are formed of spermatogonia in one or two layers, they are small in size round in shape. Primary spermatocytes are arranged in 4-8 layers in the middle zone, they are large cells with vesicular nuclei. Spermatids are the smallest spermatogenic cells near the lumen with dark nuclei.

Sertoli cells are tall with large pale nuclei and pale cytoplasm present between cells.

Also, sperms are seen in lumen. The STs are surrounded by flat myoid cells with flat nuclei (Figure 2). The interstitium contain blood vessels and groups of Leydig cells that are rounded cells with one or two vesicular nuclei and acidophilic foamy cytoplasm (Figures 1 and 2).

Group II (Metformin group) in H&E stain are showing moderate irregularity in diameter of seminiferous tubules. Many tubules are showing nearly intact layers of spermatogenic epithelium with some sperms are detected in lumen. Some tubules have disorganized germinal epithelium (Figure 3). Some spermatogenic cells have vacuolation of cytoplasm and pyknosis of nuclei (Figures 4 and 5). Degeneration of some Leydig cells and deposition of eosinophilic material in mild dilated space in between are seen (Figures 3and 5).

Group III (Sitagliptin group) in H&E stain show many deformed STs with marked irregular reduced germinal epithelium (Figure 6) Many spermatogenic and Sertoli cells show marked cytoplasmic vacuolation and pyknotic nuclei with sloughed remaining necrotic cells in wide lumina (Figures 7 and 8).

The presence of few sperms and multinucleated giant cells are noticed (Figure 9).

Wide interstitium full of acidophilic hyaline material and congested blood vessels are noticed. (Figures 6, 7 and 8).

Mallory trichrome stained sections of testes of (G I) show the tunica albuginea is formed of dense connective tissue with minimal deposition of collagen fibers in between the seminiferous tubules and basal lamina of some tubules (Figure 10). Metformin treated group (G II) show moderate deposition of collagen fibers in testicular capsule, interstitial tissue, basal lamina of some tubules and around blood vessels (Figure 11). While sections of Sitagliptin treated group (G III) show marked thickened tunica albuginea with marked deposition of collagen fibers in testicular capsule, interstitial tissue, basal lamina and around congested blood vessels (Figure 12).

Electron Microscopic Results

Electron microscope examination of the ultrathin sections of the testes of the GI show that spermatogenic epithelium are observed on fixed regular basement membrane which has myoid cell with flat oval nucleus (Figure 13). Spermatogonia cells have rounded or oval nuclei and prominent mitochondria (Figures 13 and 16). Primary spermatocytes have large euchromatic nuclei with synaptonemal complexes and a tiny rim of cytoplasm (Figures 13 and 15). Spermatids have euchromatic nuclei and peripherally located electron lucent mitochondria. Also, spermatids have acrosomal cap or vesicle or granule (Figure 15). Different parts of sperms in transverse sections show that midpieces have a central axoneme which is

surrounded by dense fibrous and mitochondrial sheaths (Figure 17).

Adjacent to the basement membrane, Sertoli cells with ovoid euchromatic indented nuclei and prominent nucleoli are detected. Their cytoplasm includes mitochondria and variable sizes, electron-dense granule and smooth endoplasmic reticulum SER cisternae (Figures 13 and 14). Tight junctions (blood-testis barrier) are observed between the adjacent Sertoli cells (Figures 14 and 16).

Interstitial Leydig cells have nuclei with a thin layer of heterochromatin . Their cytoplasm contains numerous mitochondria, a few lipid droplets, smooth endoplasmic reticulum cisternae, electron dense granules and cytoplasmic microvilli. Also, connective tissue cells and blood vessels are noticed (Figure 18).

Electron microscope examination of the testes of G II show that seminiferous tubules have thick irregular basement membrane. Nearly normal structures are recognized in spermatogonia, primary spermatocytes, spermatids and Sertoli cells. However, destructed mitochondria, vacuoles, electron dense granules and moderate dilated S.E.R cisternae in some cells are seen (Figures 19 and 20).

Some spermatids show irregular acrosomal vesicles. Others with shrunken nucleus and abnormally distributed mitochondria are detected (Figure 21). Disorganized axoneme is seen in transverse sections of the midpieces of some sperms (Figure 22).

Leydig cells contain shrunken oval nucleus, destructed mitochondria, dilated S.E.R cisternae, lipid droplets and many irregular cytoplasmic process. Congested dilated vessels is also seen in C.T. (Figure 23)

Electron microscope study of the testes of G III show that the STs are enveloped by an extremely thick irregular basement membrane. Myoid cells appear with irregular heterochromatic nuclei (Figure 24).

Spermatogonia, primary spermatocytes and Sertoli cells have different degree of degeneration. Their cytoplasm shows many ballooned and damaged mitochondria, vacuolation and dilated SER cisternae. Discontinuous interrupted blood testis barrier is also observed (Figures 24, 26 and 27). Spermatid show irregularly notched nucleus covered by irregular acrosomal cap with irregularly distributed damaged intracytoplasmic mitochondria (Figure 25).

Nearly all mid pieces of sperms show markedly affected distorted axoneme, fibrous, and mitochondrial sheaths in the transverse sections (Figure 28). Leydig cells show shrunken nucleus, dilated S.E.R cisternae, damaged mitochondria, many vacuoles, and variable-size electron-dense granules. Enlarged congested blood vessels and C.T. infiltration cells are also observed (Figure 29).

Immunohistochemical Results

Proliferating cell nuclear antigen (PCNA)

The nuclei of spermatogonia and primary spermatocytes are the main sites for positive PCNA reaction. Strong positive cells are evident in the control (group I) (Figure 30). The testicular sections of GII reveal moderate positive nuclear immunoreactions for PCNA in germ cells nuclei (Figure 31). While, (G III) sections expose weak positive reaction in germ cells nuclei. (Figure 32).

Androgen receptor (A.R.)

Nuclei of Sertoli cells, Leydig cells, and myoid cells show strong positive staining in control group (Figure 33). In (G II), moderate positive nuclear AR reaction is seen in the same cells (Figure 34). While, in (G III) weak positive nuclear AR reaction is seen (Figure 35).

CASPASE-3

Sections of the G I reveal mild caspase-3 cytoplasmic staining (Figure 36), while G II show moderate positive reaction for caspase -3 (Figure 37). On the other hand, intense positive expression of caspase-3 in G III is seen. (Figure 38).

Morphometric analysis

The mean diameter of STs, height of spermatogenic epithelium, number of Sertoli cells show a non-significant change in (G II) in compare to control (p>0.05) (Table 2 and histograms 5,6 &7). whilst, mean number of PCNA positive cells and AR positive cells show significant decrease and caspase-3 positive cells show significant increase as referred to control ($P < 0.05$) (Table 2 and histograms 8, 9 &10).

While, (G III) show a highly significant decrease in all these parameters and highly significant increase in caspase-3 as referred to control group (P<0.001) except the mean number of Sertoli show significant decrease as referred to control (P<0.05) (Table 2 and histograms 5,6,7,8, 9 &10).

Table 1: Mean value ± SD of biochemical parameters in all studied groups

Parameters	Testesterone (mg/d)	MDA (n mol/g)	Sperm count (10^6/mm^3)	Sperm motility (%)
Control group	4.510±0.351	112.670±8.210	69.000±2.670	80.000±7.410
Metformin group	2.920±0.781*	132.240±5.140*	58.770±5.780*	72.600±0.960
Sitaglipitin group	0.945±0.135**	218.020±7.150**	26.500±1.260**	24.000±7.500**

* Significant (p<0.05) as compared to Control group.
** highly significant (p<0.001) as compared to Control group.

Table 2: Mean value ± SD of morphometric parameters in all studied groups

| Parameters	ST diameter (μm)	GE height (μm)	sertoli	PCNA	AR	Caspase
Control group	468.104±7.852	148.121±4.214	17.450±1.218	4.153±1.312	29.000±4.700	0.102±0.011
Metformin group	450.650±29.598	125.441±7.343	15.160±1.515	3.029±0.741*	23.000±8.200*	0.367±0.027*
Sitaglipitin group	401.015±16.318**	100.521±3.246**	10.500±1.522*	1.452±0.689**	13.000±5.100**	0.805±0.014**

* Significant ($p<0.05$) as compared to Control group.
** highly significant ($p<0.001$) as compared to Control group.
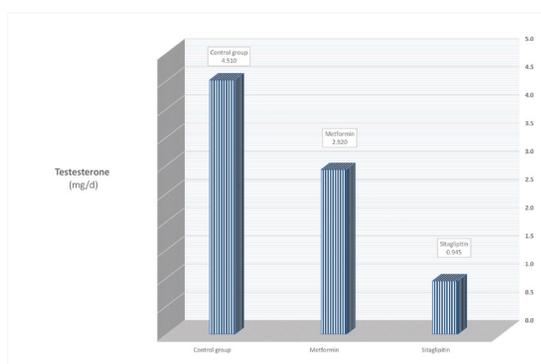
Histogram 1: The serum Testosterone level of various experimental groups.

Histogram 2: The MDA level of various experimental groups

Histogram 3: The sperm count of various experimental groups.

Histogram 4: The sperm motility of various experimental groups.

Histogram 5: Seminiferous tubules diameter of various experimental groups.
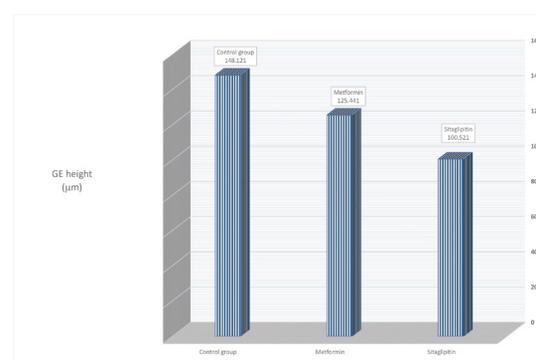
Histogram 6: The height of germinal epithelium of various experimental groups.
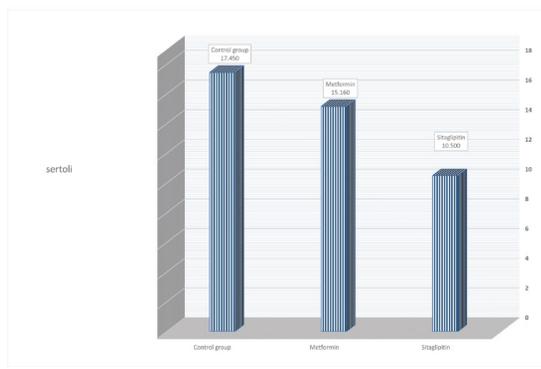
Histogram 7: The number of Sertoli cells of various experimental groups.
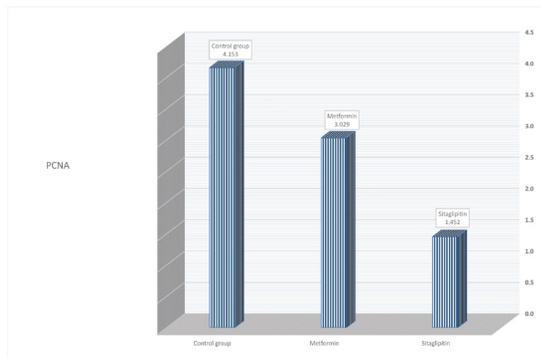
Histogram 8: The area percentage of PCNA immunoexpression of various experimental groups.
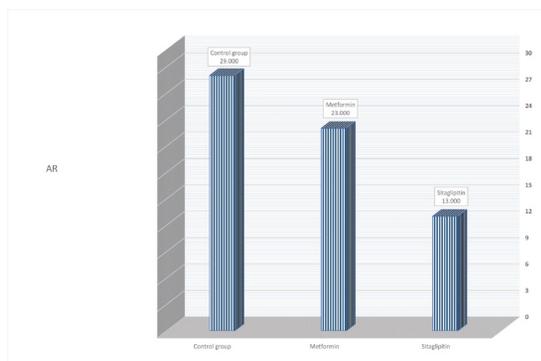
Histogram 9: The area percentage of AR immunoexpression of various experimental groups.
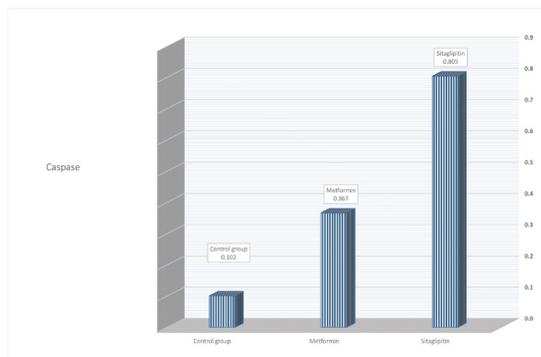
Histogram 10: The area percentage of caspase -3 immunoexpression of various experimental groups.

Fig. 1: H&E stained testis section of control group showing the seminiferous tubules (St) lined with spermatogenic epithelium and sperms (Sz) in the lumen. The interstitial (L) Leydig cells with acidophilic cytoplasm are seen. (X100)
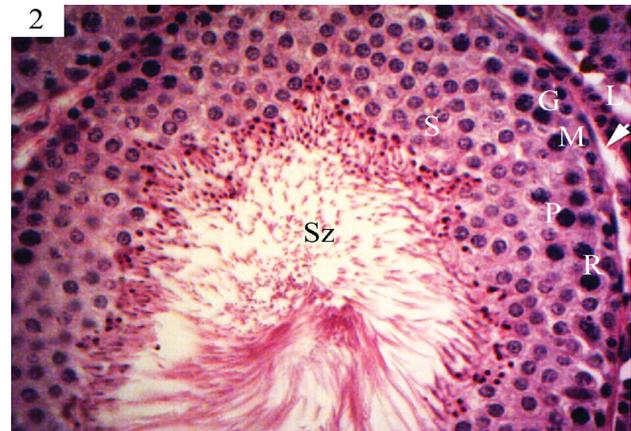
Fig. 2: H&E stained testis section of control group showing the seminiferous tubule lined with spermatogonia (G), primary spermatocytes (P), spermatids (S), and Sertoli cells (Se) with fully packed sperms (Sz) in the lumen. The interstitium has Leydig cells with acidophilic cytoplasm (L). Notice myoid cells with flattened nucleus (My) and regular thin basement membrane (arrow head). (X400)
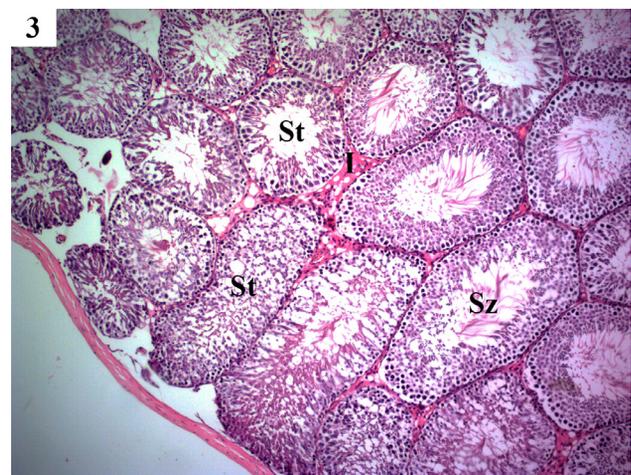
Fig. 3: H&E stained testis section of Metformin group showing: Seminiferous tubules (St)with nearly intact layers of spermatogenic epithelium with moderate deposition of eosinophilic hyaline material in the interstitial space (I) Notice: presence of sperms in lumen of some tubules (Sz). (X 100)
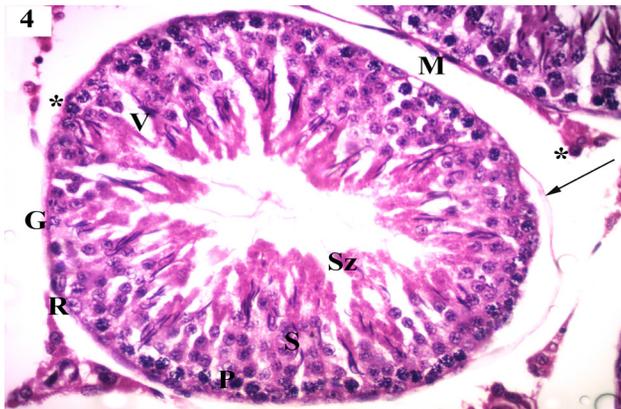
Fig. 4: H&E stained testis section of Metformin group showing Seminiferous tubule with spermatogonia (G), primary spermatocytes (P), spermatids (S), Sertoli cells (Se), and few sperms in lumen (Sz). Some cells with pyknotic nuclei (*) and vacuolation(V) are noticed. Empty spaces between germ cells are present. Myoid cells with flat nuclei (My) & irregular partially separated basement membrane (arrow) are seen. (X400).
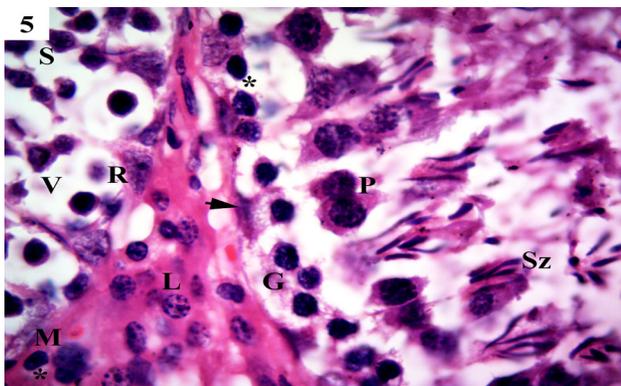

Fig. 5: H&E stained testis section of Metformin group showing parts of two seminiferous tubules with spermatogonia (G), primary spermatocytes (P), spermatids (S), apparently normal some Sertoli cells (Se) and other degenerated cells (arrow head) are seen. Few sperms are seen in lumen (Sz). Pyknotic nuclei (*), vacuolation(V) of cytoplasm and myoid cells with degenerated nuclei (My) are seen. Leydig cells (L) are seen between tubules and some of them are pyknosed (*). (1000).
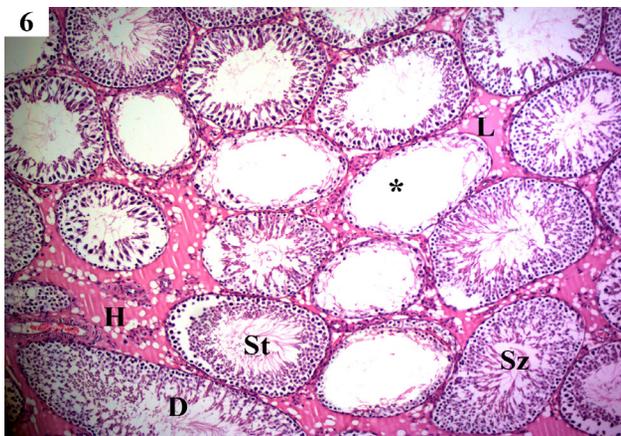

Fig. 6: H&E stained testis section of Sitagliptin group showing irregularity in diameter of seminiferous tubules (St) , some showing dilated lumen (D) and others, completely damaged tubule (*). Sperms in lumen of some tubules (Sz) , wide spaces with eosinophilic hyaline material (H) and degenerated Leydig cells between tubules (L). are seen (x 100)
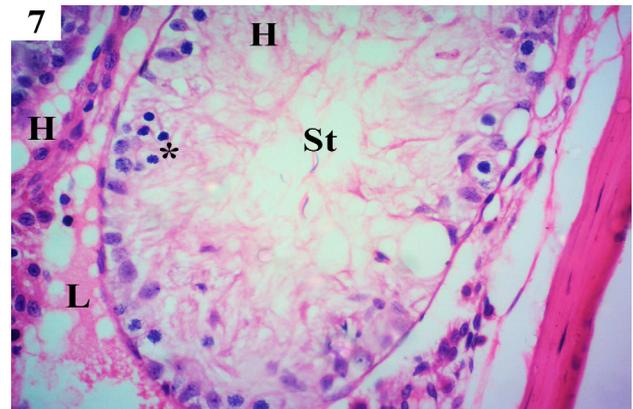

Fig. 7: H&E stained testis section of Sitagliptin group showing marked loss of spermatogenic epithelium of seminiferous tubules (St), some remaining cells showing pyknosis (*) and hyalinized material(H) inside and between seminiferous tubules. Some Leydig cells (L) are degenerated and others are pyknotic. (x 400)
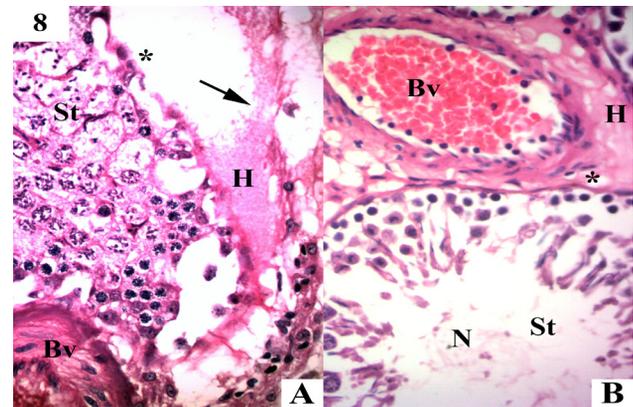

Fig. 8: H&E stained testis section of Sitagliptin group showing: (8A) marked irregular spermatogenic epithelium separated from basement membrane (arrow) of seminiferous tubules (St). (8B) showing irregular tubules (St) with disorganized spermatogenic epithelium and sloughed necrotic material in the lumen (N). Notice: congested blood vessel (Bv) and hyalinized eosinophilic material (H) and some cells showing pyknosis (*) in (8A & 8B) (x 400)
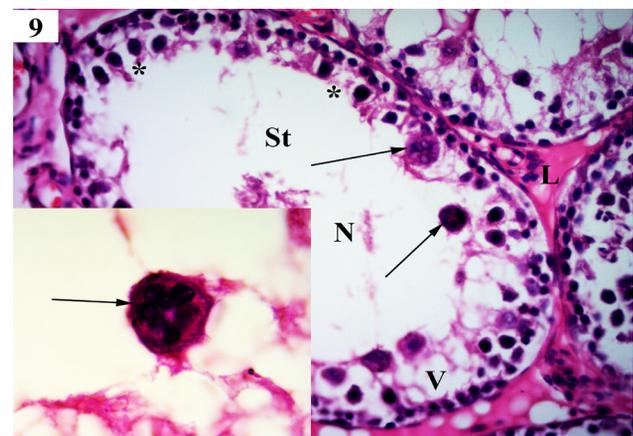

Fig. 9: H&E stained testis section of Sitagliptin group showing: loss of most layers of germinal epithelium of seminiferous tubules (St), vacuolation of cytoplasm (V), pyknosis of some nuclei of spermatogonia (*), degenerated Leydig cells (L) and no sperm in lumen. Many giant cells (arrow), sloughed necrotic material(N) in the lumen are seen (Hx& E x 400). Inset showing magnified giant cell (x 1000)
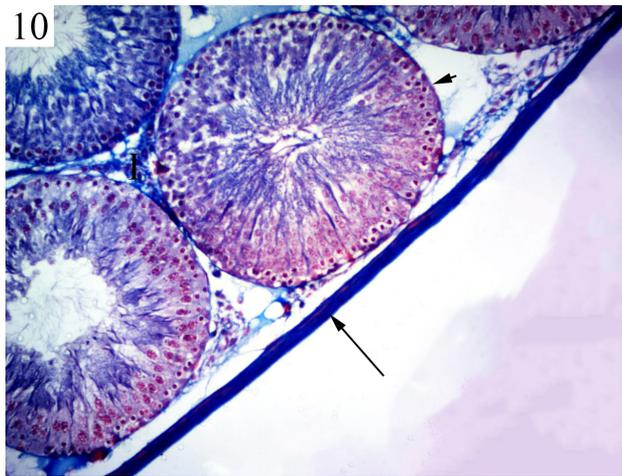
Fig. 10: Mallory trichrome stained section of control group showing: minimal collagen fibers in testicular capsule (arrow), interstitial tissue (I) and basal lamina (arrow head). (X200)
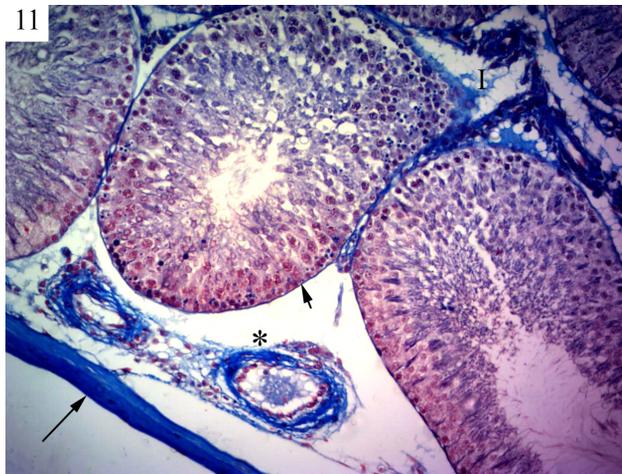

Fig. 11: Mallory trichrome stained section of Metformin group showing moderate collagen fibers in testicular capsule (arrow), interstitial tissue (I), basal lamina (arrow head),and around congested blood vessels (*). (X200)
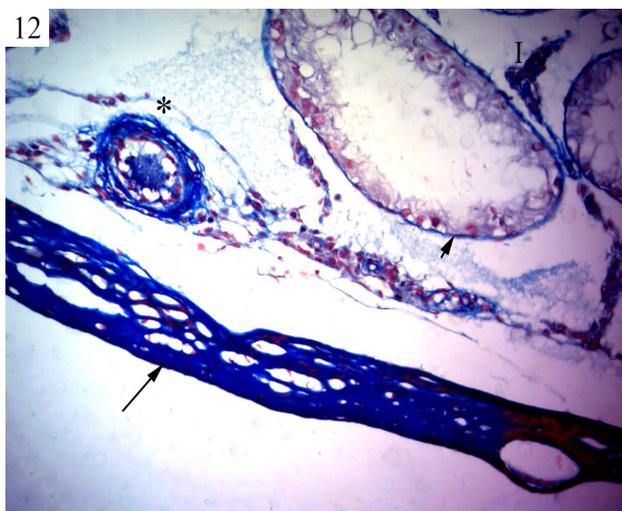

Fig. 12: Mallory trichrome stained section of Sitagliptin group showing marked collagen fibers in testicular capsule (arrow), interstitial tissue (I), basal lamina (arrow head) and around congested blood vessels (*) (X 200)
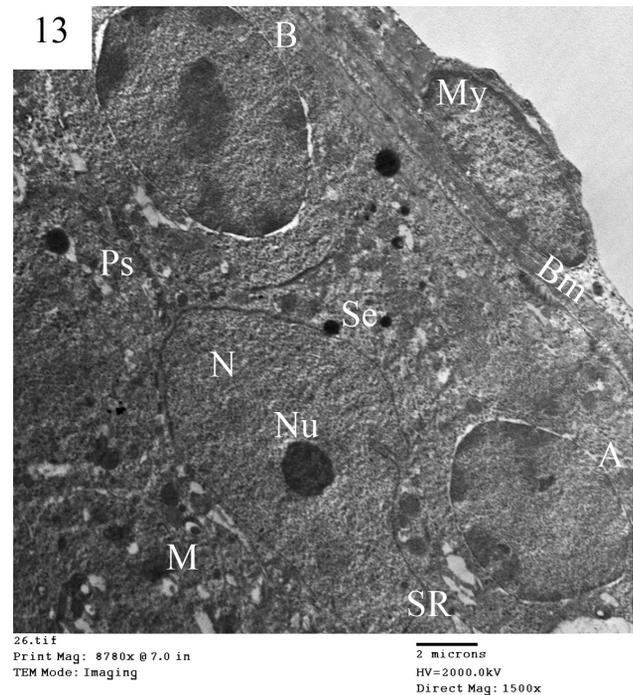

Fig. 13: Electron micrograph of part of seminiferous tubule of (G1) showing : basement membrane (Bm) , myoid cells (My) with oval nucleus , spermatogonia A (A) with rounded euchromatic nucleus, spermatogonia (B) with dark stained oval nucleus , primary spermatocyte with large granular nucleus (Ps), Sertoli cell (Se) with oval intended nucleus (N) and prominent nucleolus (NU). Notice: mitochondria (M) & prominent SER (SR) are seen.
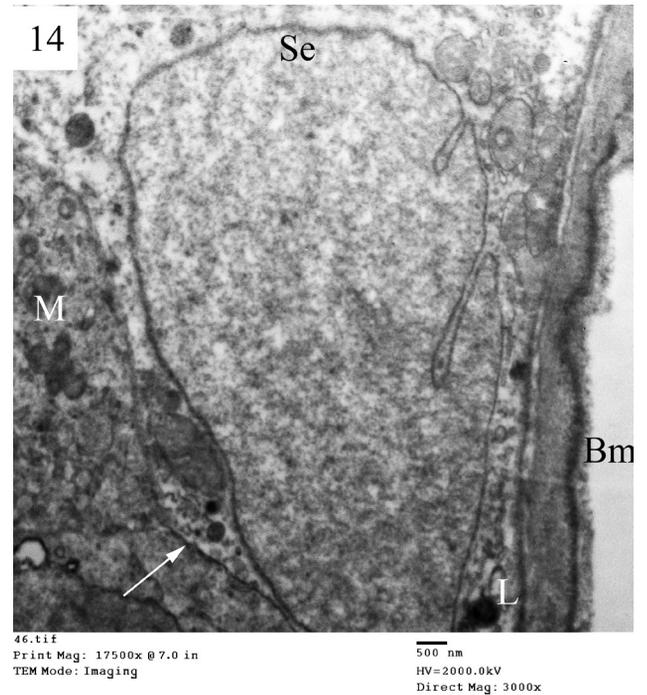

Fig. 14: Electron micrograph of part of seminiferous tubule of (G1) showing: basement membrane (Bm), Sertoli cell (Se) with oval intended nucleus and electron-dense granules. Notice: mitochondria (M), lysosome(L) and clear blood testis barrier (arrow) are seen.
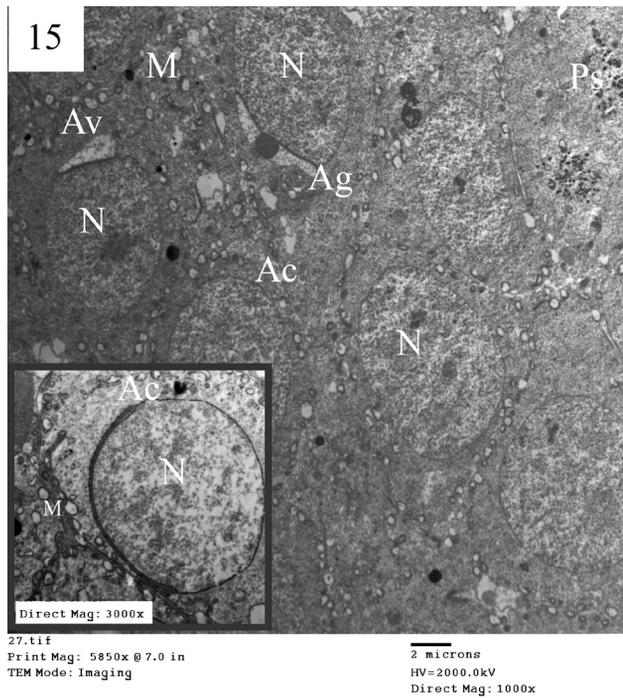
Fig. 15: Electron micrograph of spermatid cells of (G1) showing oval euchromatic nucleus (N) covered by acrosomal cap (Ac), acrosomal vesicle (Av), acrosomal granule (Ag), and peripherally located electron lucent mitochondria (M). primary spermatocyte (Ps) with electron dense chromatin are seen. Inset showing higher magnification of spermatid nucleus with acrosomal cap
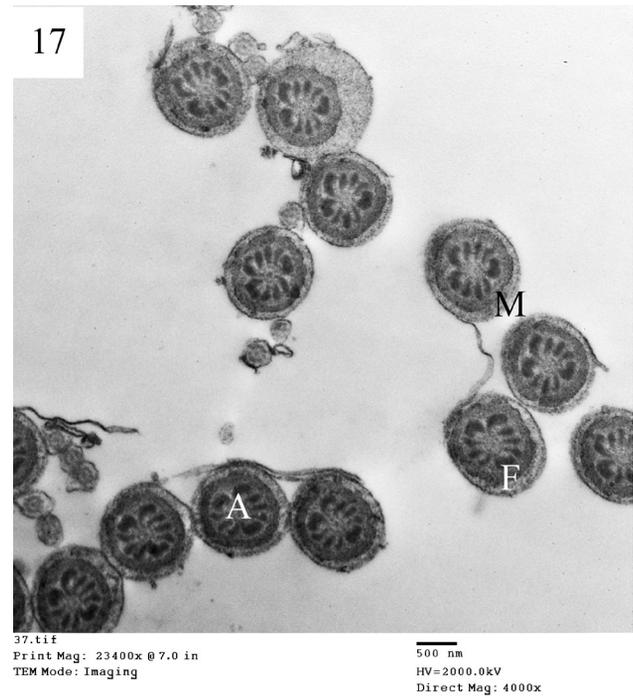
Fig. 17: Electron micrograph of mid piece of sperms of(G1) showing axoneme (A), fibrous sheath (F), mitochondrial sheath(M)
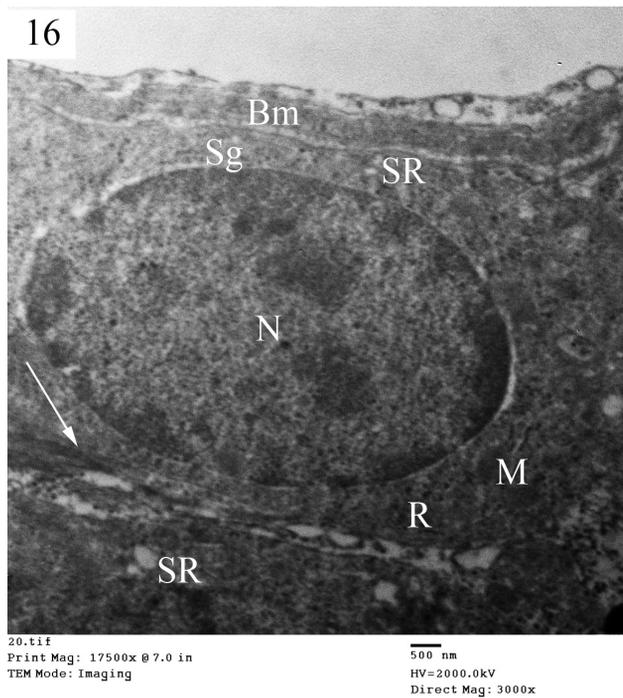
Fig. 16: Electron micrograph of spermatogonia (Sg) of testis (G1) resting on basement membrane (Bm) with large oval euchromatic nucleus (N), cytoplasm showing SER (SR), mitochondria (M), free ribosomes (R). Notice: Blood testis barrier (arrow)
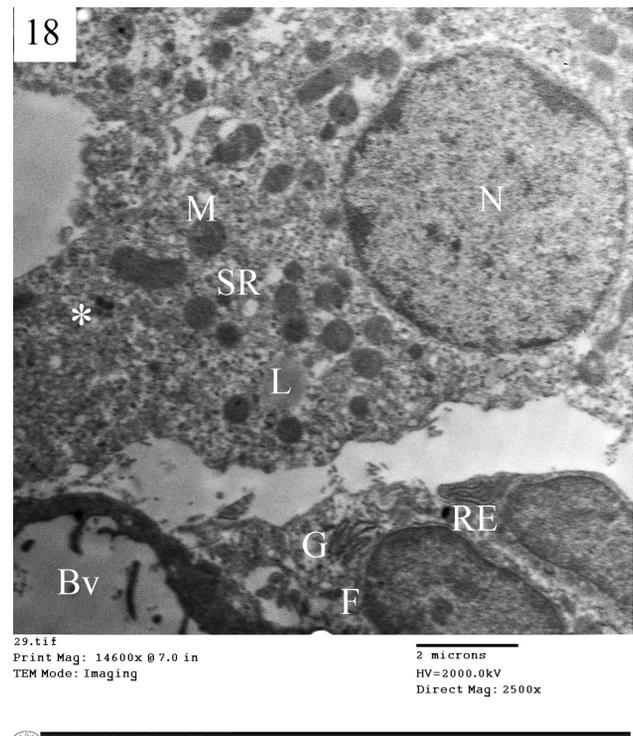
Fig. 18: Electron micrograph of Leydig cells of testis of G1 showing: rounded euchromatic nucleus with peripheral hetero chromatin (N) , multiple mitochondria (M) , lipid droplets (L), S.E.R cisternae (SR), electron dense granules (*), blood vessels (Bv). Fibroblast (F) with prominent Golgi (G) and R.E.R (RE) is seen.
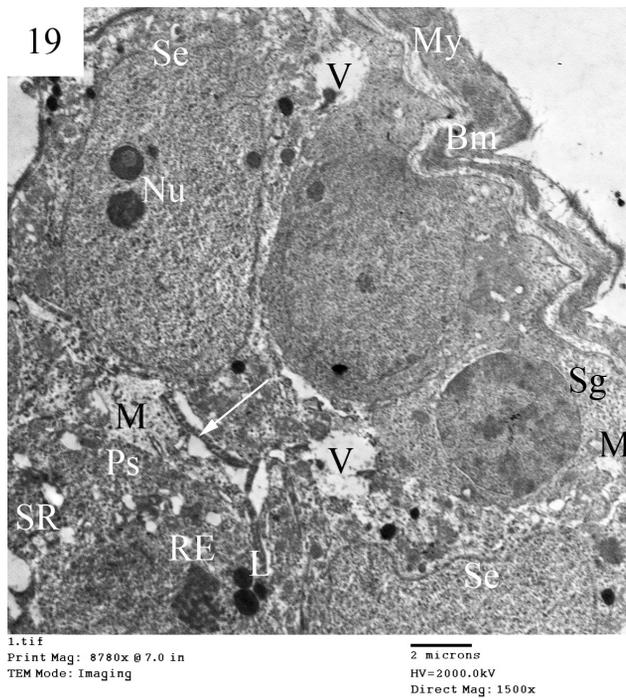
Fig. 19: Electron micrograph of part of seminiferous tubule of testis of (GII)showing: thickened irregular basement membrane (Bm), part of degenerated myoid cells (MY), spermatogonia (Sg) with small hetero chromatic nucleus and vacuolated cytoplasm, primary spermatocyte (Ps) with destructed mitochondria (M), lysosomes (L) and vacuolation (V) and RER (RE) and dilated SER (SR) are seen. Sertoli cells (Se) with intended nucleus, two nucleoli (Nu) and irregular discontinued blood testis barrier (arrow) are seen
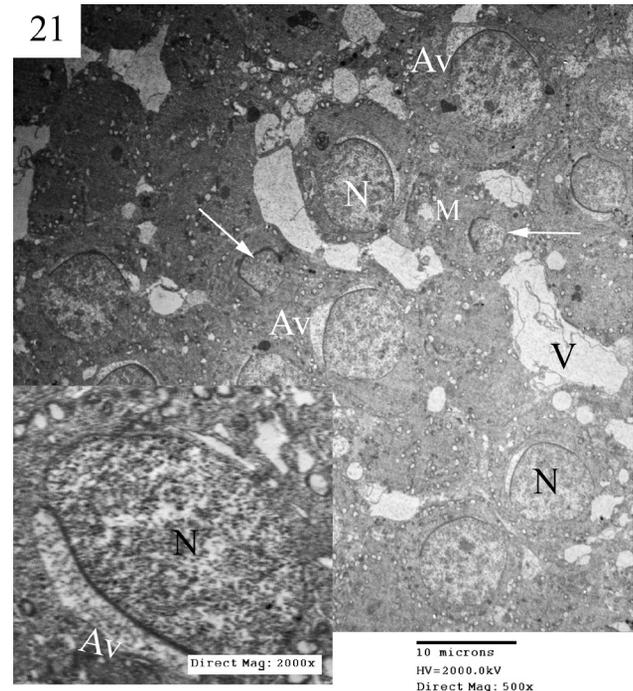
Fig. 21: Electron micrograph of multiple nuclei (N) of spermatids of testis of GII showing irregularity in acrosomal vesicle (Av) of some spermatids, shrunken irregular nucleus of other spermatids (arrow), wide spaces between cells (V) and degenerated mitochondria (M). Inset showing oval nucleus (N) covered by irregular chromosomal vesicle (Av),
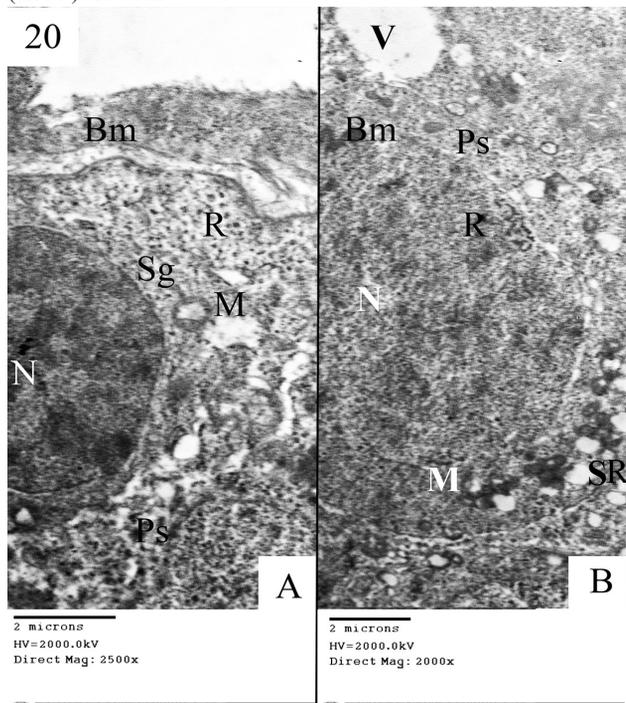
Fig. 20: Electron micrograph of testis of G II showing (A) spermatogonia (Sg) resting on irregular basement membrane (Bm) having nucleus with hyper chromatin (N), damaged mitochondria (M), ribosome(R). (B) showing primary spermatocyte (Ps) with: large nucleus (N). Vacuolation of cytoplasm (V) and dilated SER (SR) can be seen in (A&B)

Fig. 22: Electron micrograph of mid piece of tail sperms of G II showing disorganized axoneme (A), fibrous sheath (F), mitochondrial sheath(M) of some sperms. Others areapparently normal(arrow)
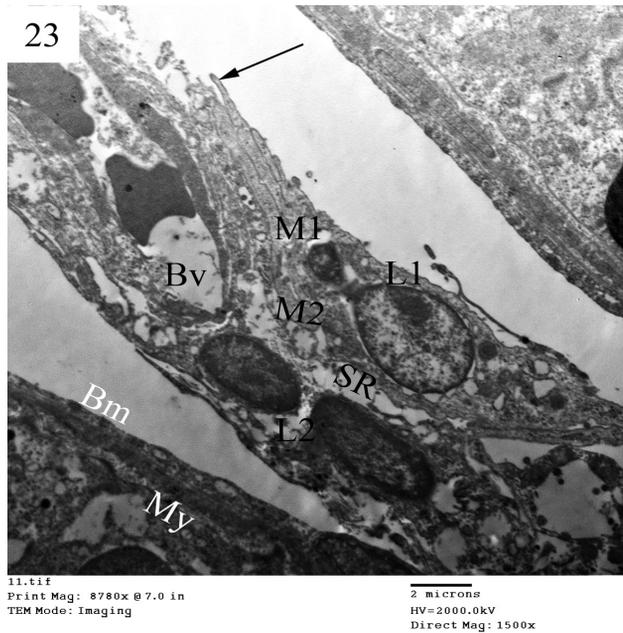
Fig. 23: Electron micrograph of multiple Leydig cells of testis of GII showing cell with peripheral hyperchromatism in nucleus (L1), other 2 cells showing shrunken hyperchromatic nuclei (L2) mitochondria with destructed cisternae (M1) & ballooned damaged mitochondria (M2) dilated S.E.R cisternae (SR), multiple cytoplasmic processes (arrow), congested blood vessel (Bv) are seen Notice: adjacent seminiferous tubule showing some degeneration with degenerated basement membrane(Bm) & myoid cell (My)


Fig. 24: Electron micrograph of testis of GIII showing markedly thickened irregular basement membrane (Bm) & degenerated myoid cell (My), dark hyperchromatic shrunken spermatogonia (Sg). Sertoli cell (Se) with shrunken irregular nucleus (N), marked vacuolation of cytoplasm (V), dilated (SR), damaged mitochondria (M). primary spermatocyte (Ps) with enlarged nucleus with loss it's synaptonemal complex (granular chromatin) (n) Notice: remnant of blood testis barrier (arrow).

Fig. 25: Electron micrograph of spermatid of testis of G III showing irregularly notched nucleus (N) covered by irregular acrosomal cap (Ac), marked ballooned irregularly distributed mitochondria (M) Vacuolation of cytoplasm (V), dilated SR , lysosome(L) and residual body are seen (arrow).
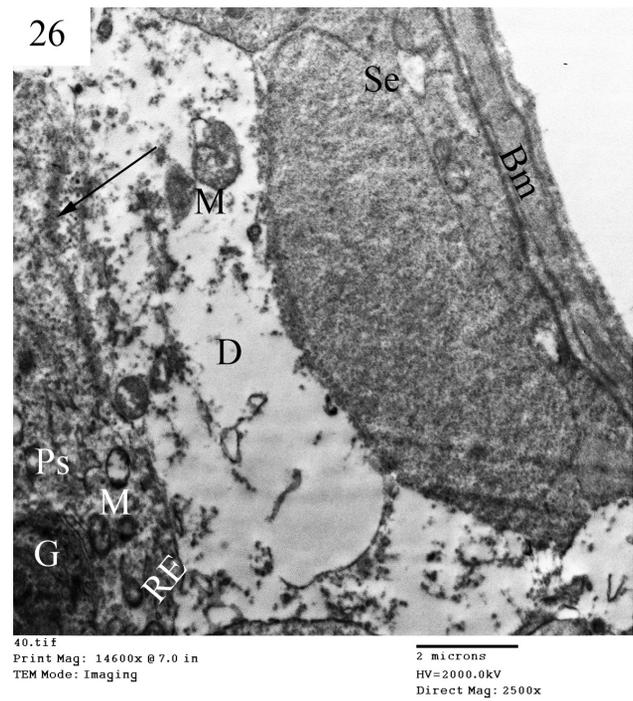
Fig. 26: Electron micrograph of Sertoli (Se)cell of testis of GIII resting on basement membrane (Bm) with elongated nucleus, degenerated vacuolated cytoplasm (D), damaged mitochondria (M), destructed barrier (arrow), primary spermatocyte (Ps), RER (RE) and prominent Golgi (G) apparatus are seen.
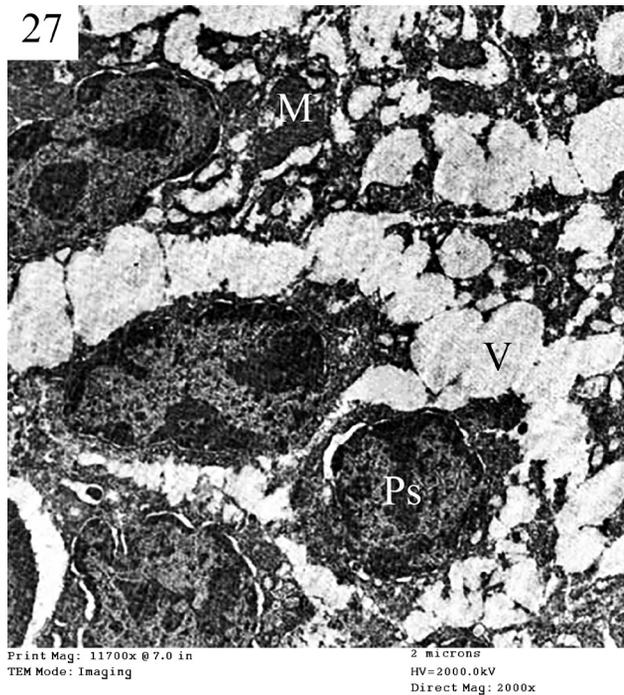
Fig. 27: Electron micrograph of degenerated primary spermatocyte (Ps)cells of testis of GIII showing degenerated vacuolated cytoplasm (V), damaged mitochondria (M).
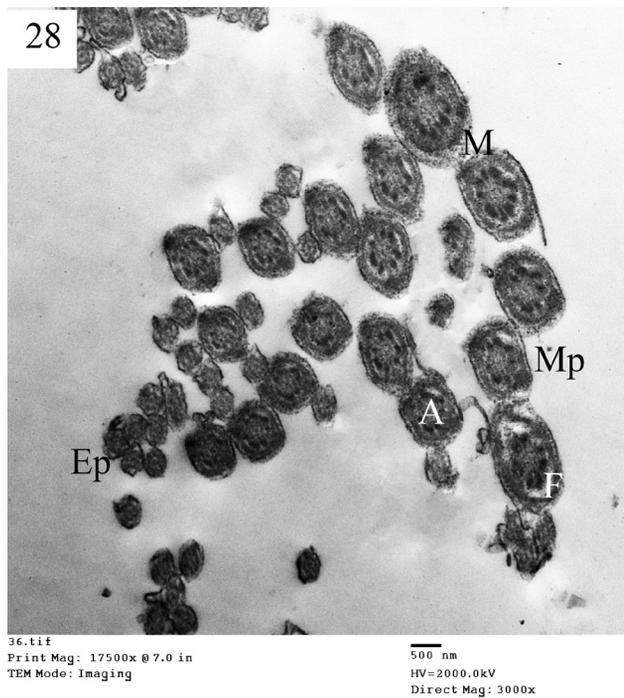
Fig. 28: Electron micrograph of sperms of (GIII) showing markedly disorganized axoneme (A), fibrous sheath(F), mitochondrial sheath(M) of mid piece (Mp) and distorted axoneme of end piece (Ep)
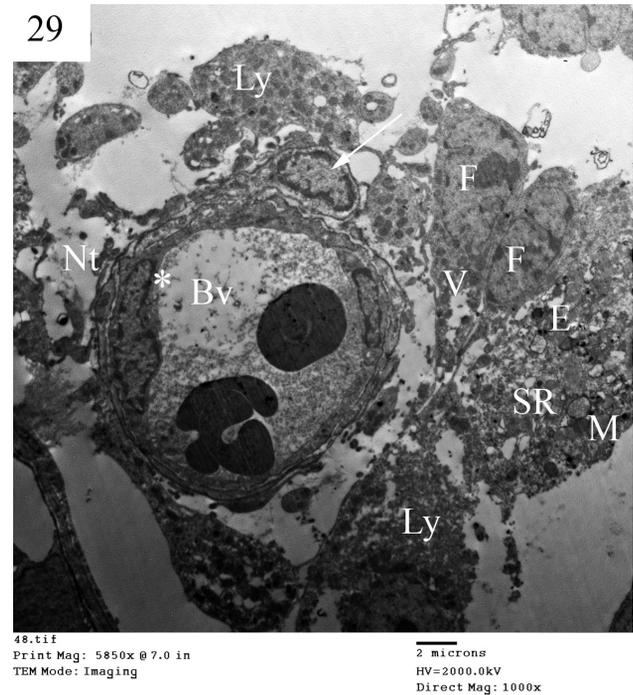
Fig. 29: Electron micrograph of interstitium of testis of GIII showing: degenerated Leydig cells (Ly), vacuolation of cytoplasm (V), electron dense bodies (E) dilated SR and damaged mitochondria (M).Enlarged congested blood vessels (Bv) lined by endothelium cells (*) and surrounded by pericyte (arrow), degenerated connective tissue cells (F) and necrosed tissue (Nt) are seen.
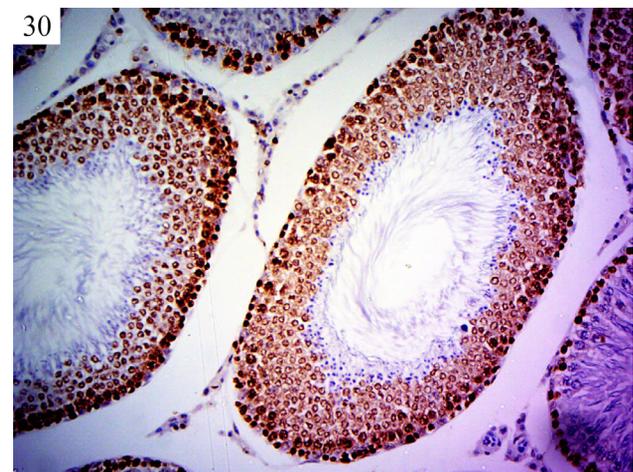
Fig. 30: PCNA stained testis section of control group showing strong positive immunostaining in most nuclei of the basal germinal cells in seminiferous tubules. (X200)
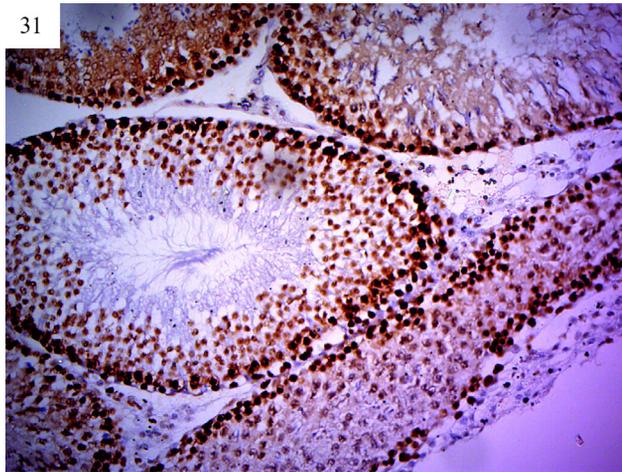
Fig. 31: PCNA stained testis section of Metformin group showing moderate positive immunostaining in nuclei of basal germ cells in seminiferous tubules. (X200)
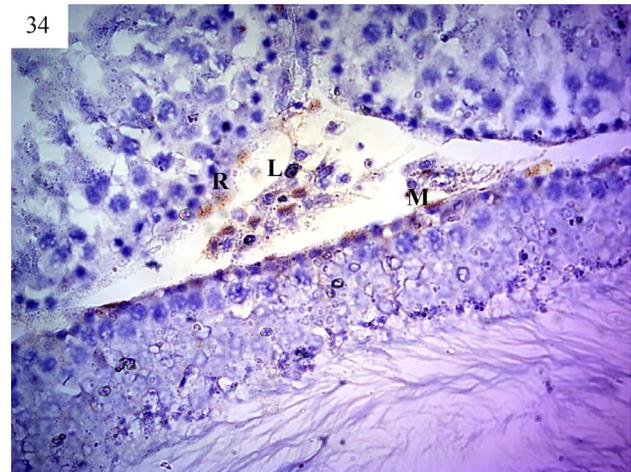
Fig. 34: Androgen Receptor stained testis section of Metformin group showing moderate nuclear immunoexpression in interstitial Leydig cells (L), myoid cells (My) and sertoli cells (Se). (X 400)
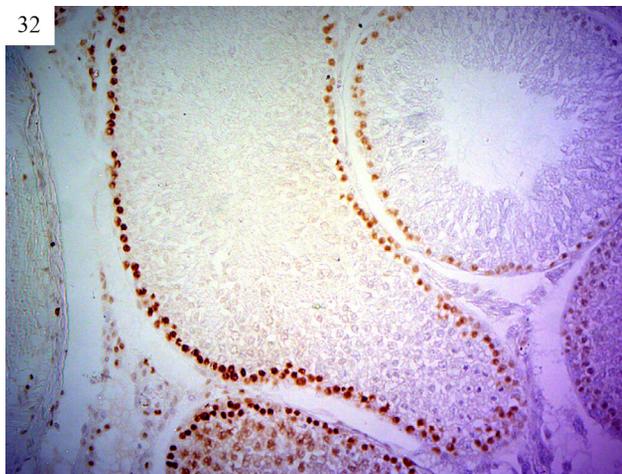
Fig. 32: PCNA stained testis section of Sitagliptin group showing weak positive immunostaining in nuclei of basal germ cells in seminiferous tubules. (X200)
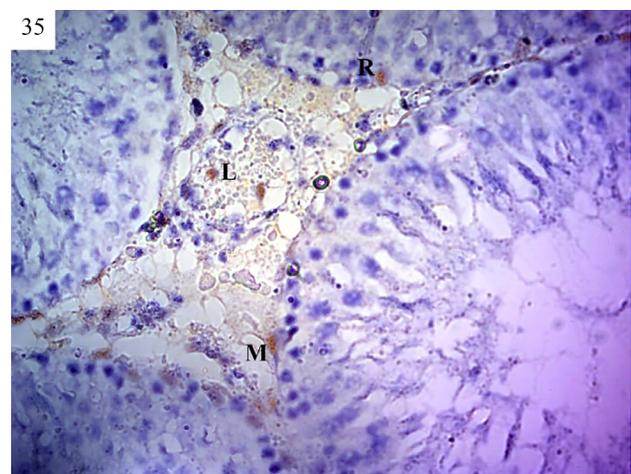
Fig. 35: Androgen Receptor stained testis section of Sitagliptin group showing weak nuclear immunoexpression in interstitial Leydig cells (L), myoid cells (My) and sertoli cells (Se). (X 400)
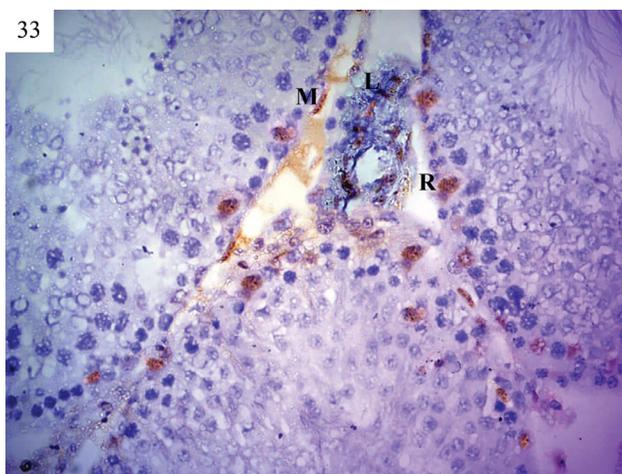
Fig. 33: Androgen Receptor stained testis section of control group showing strong nuclear immunoexpression in interstitial Leydig cells (L), myoid cells (My) and sertoli cells (Se). (X 400)
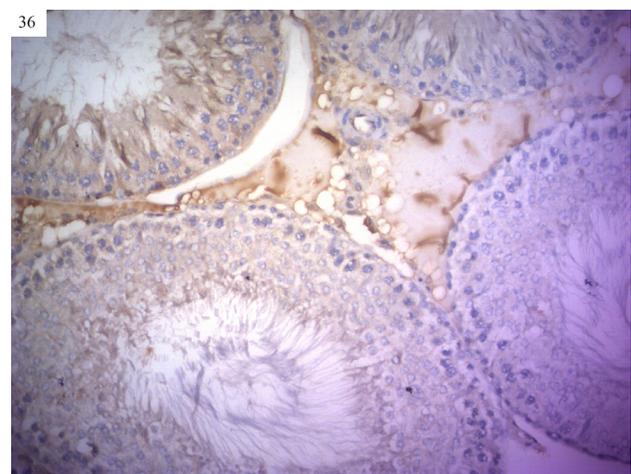
Fig. 36: Caspase-3 stained testis section of control group showing mild cytoplasmic immunostaining. (x 200)
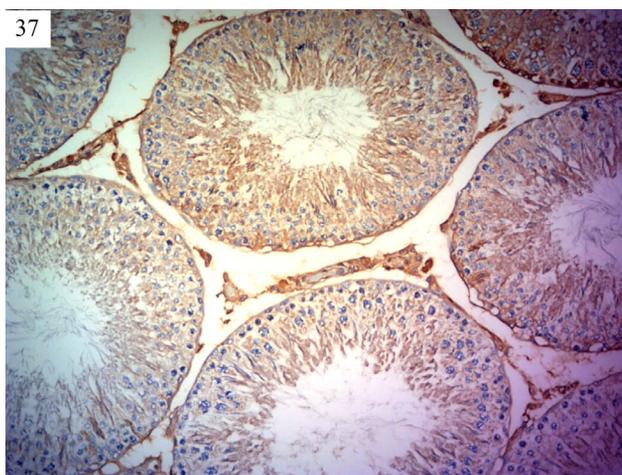
Fig. 37: Caspase-3 stained testis section of Metformin group showing moderate cytoplasmic immunostaining. (x 200)
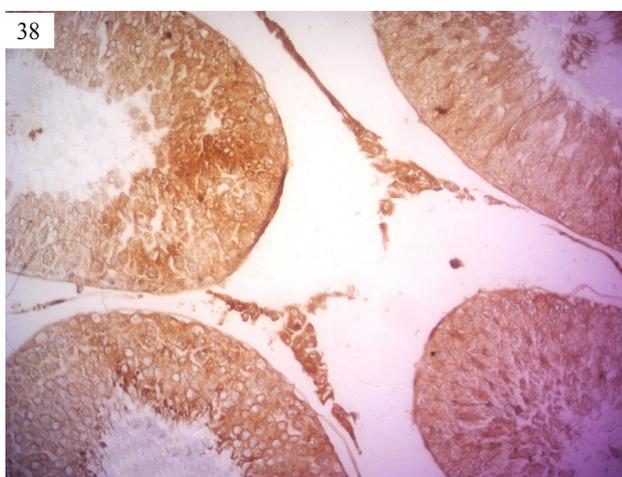
Fig. 38: Caspase-3 stained testis section of Sitagliptin group showing intense positive cytoplasmic immunostaining. (x 200)

DISCUSSION

At the last three decades, a widespread raise in diabetic patients particularly T2DM has been discovered especially in developing countries[22]. The global ratio of T2DM in the people aged 20-79 years is expected to extend from 8.3% (382 million patients) in 2013 to 10.1% (592 million patients) in 2035[23]. Metformin (dimethyl biguanide) is the first-line medication treatment for T2D. Worldwide, more than100 million patients are prescribed for this medication annually[24] Sitagliptin is a selective, oral, DPP-4 inhibitor that enhance glycemic control in T2DM disease[25].

The present study is the first to illustrate the effects of Metformin and Sitagliptin on the testicular tissue, as an essential reproductive endocrine organ. The MDA is a frequent and reliable marker that used to determine oxidation stress (OS)[26]. It causes peroxidation of polyunsaturated fatty acids (PUFA)[27]. and subsequently loss of membranes structures and functions[28]. Testes, being rich in PUFA and having high rates of metabolism, cell replication and poor antioxidant defenses, are much more vulnerable to peroxidation injury[29].

In this work, statically elevated MDA in (GIII) Sitagliptin rats might explain marked histo¬logical changes. Many of STs are distorted with irregular disorganized epithelium and desquamated cell remnants within their lumina. These are in harmony with Ayuob *et al.,*[30] who explains these changes by increased levels of MDA. This is supported by morphometric results as statically significant highly decrease in diameter of STs and height of lining spermatogenic epithelium and statically biochemical results as count nd motility of sperms and level of TH as referred to the control[30] confirm histological results in this group.

This is enforced by the ability of MDA to react with many molecules as proteins or DNA leading to damage of DNA, arresting cell cycle, apoptosis of germ cell and consequently low sperm[27]. The high susceptibility of spermatozoa to DNA damage is attributed to cytoplasm loss that includes anti-oxidant enzymes after spermiation[31].

Interestingly, statically significant Sertoli cell affection in (G III) lead to decrease in seminiferous tubule fluid that contains nutritional and hormonal factors needed for spermatogenesis[32]. Affected Sertoli cells result in metabolic disturbance in germ cells, vacuolation of their cytoplasm, death and shedding as similar observations are detected by previous investigators[33].

These are in harmony with electron microscopic results that show many vacuoles which are initiated from fusion and dilation of cytoplasmic organelles as SER[25,26]. Also, decreasing in number of Sertoli cells can be explained by suppression of follicle-stimulating hormone (FSH) secretion by the pituitary or decreasing its sensitivity at the gonad level. These are in harmony with Kayampilly and Menon[34]. Sertoli cells form important factors for preservation of the Leydig cells. So, Leydig cells also exhibit degenerative changes which are confirmed by ultrastructural results of irregular nuclei and destructed mitochondria.

Homogenous acidophilic material between some STs of (G III) can be due to impaired phagocytic function of Sertoli cells, so hyalinization of the degenerated germ cells is noticed[35]. In addition, Agarwal and Said[36] suggest that chemical mediators are emitted shortly following tissue destruction, raising the blood vessels wall permeability and causes plasma infiltration and fluid exudates to the surrounding tissue. The testicular blood vessels are dilated and congested. It may be explained by oxidation stress[30]. Multinucleated giant cells are discovered in the lumen of STs of this group which pointing to inability of tetraploid primary spermatocytes to finish the meiotic division or fusion of spermatids after the failure of intercellular bridges breakdown at early phases of spermiogenesis and are explained by excessive apoptosis due to OS via mitochondrial dysfunction[37].

Moreover, some authors suggest that multinucleated giant cells may be caused by aggregation of the sloughed degenerated spermatocytes and spermatids[38]. These results are supported by the electron microscopic examination

which show damaged various germ cells and cytoplasmic vacuolation in group III and is also enforced by marked statically increased of caspase 3 immunostaining in this group.

Apoptosis can be explained by elevated oxidative stress either due to elevated free radical formation or due to suppression of antioxidant defenses[39]. Zhang *et al.*[39] explains that, elevated ROS disturb the inner and outer mitochondrial membranes, liberating of cytochrome-C protein, which stimulates caspases and makes apoptosis[33]. As well, Fenech *et al.,* add that genetic defects in cell cycle checkpoints or DNA repair genes occur after oxidative stress leading to apoptosis[40].

Sertoli (SCs) cells are key regulators of the two basic functions of the adult testis which are spermatogenesis and androgen secretion. Also, SCs work on the integrity of germinal epithelium. Impaired spermatogenesis and the subsequent decrease in height of spermatogenic epithelium and diameter of STs are noticed in G III and confirmed by morphometrical study. This may lead to widening of peritubular tissue. These findings are confirmed by Ayala[27].

Detached spermatogenic epithelium and presence of exfoliated germ cells remnants in STs lumen in (G III) could be due to the disturbance of particular connect between developing germ cells and Sertoli cells upon OS[41,42]. Morphometrically, there is significant decrease in number of Sertoli cells in G III as compared to the control, this finding is in accordance with Rato[41]. Significant decreasing in sperm motility and count are showed in (G III) ,similar features are reported by Kao *et al.,*[43].

Reduced motility can be explained by elevated ROS and free radicals[44]. Serum testosterone level in group III show marked significant decrease and could be attributed to Leydig cells disturbances due to oxidative stress. OS can alter protein kinase C , that is an important factor in steroidogenesis in Leydig cells[45]. It suppresses mRNA for P450 cholesterol side chain lyase (the key enzyme)[46]. In addition, it is reported that, decreasing in the testosterone level lead to breakdown of seminiferous tubules and damage of germ cells[47]. Also, the study of Sasso-Cerri and Cerri[42] add that decreased intratesticular testosterone is accompanied with irregular vimentin filaments of Sertoli cells leading to germ cells lost This is confirmed by statistical results which show significant reduction in testosterone, sperm numbers and motility..

The H&E results are confirmed by electronic microscopic findings which add distortion of blood-testis barrier and consequently separation of immature germ cells[48].

ARs exist in Leydig, Sertoli and myoid cells only. The statistical results of nuclear immunoreactivity in this group is decreasing significantly due to destruction of these cells[32].

In Mallory trichrome stained sections of the present study, show excessive deposition of collagen fibers around and in between the seminiferous tubules in GIII. These results are proved by many previous studies[49] which have reported that many factors that release from chronic inflammatory cells can initiate the process of fibrosis. In addition, it can be mediated through epithelium transformation into another type of tissue e.g. connective tissue in response to severe injury and this phenomenon is called epithelial mesenchymal transformation. This transformation is done by inflammatory cells (fibroblasts)[29].

DNA replication, excision and repair can be mediated mainly by nuclear polypeptide (PCNA). In the present study, the significant statically decrease in PCNA immunostaining in GIII as compared with the control group may indicate that Sitagliptin has an inhibitory effect on the proliferating action in the germ cells. This is confirmed by Annangi[50] who explained it by activation of 5' adenosine monophosphate-activated protein kinas (AMPK).

MET increases free radicals causing lipid peroxidation and changes the defense systems of body tissues and increases ROS leading to oxidation stress. This can be accounted for declining in the testicular Glutathione (GSH) levels. GSH is a tripeptide, powerful nucleophile and major defense against cellular destruction by free radical[51].

In the current work, statistically significant increase of concentration of MDA in testicular tissues of (G II) can confirm oxidation stress by MET.

H&E examination of Metformin treated group (G II) reveal irregularity in diameter of a few seminiferous tubules, some degeneration, pyknosis and few vacuoles. This is in agreement with Tartarin[52] who add that, suppression of Sertoli cell can be mediated by Metformin in a dose-dependent manner in mice cultures.

The ultrastructural results in (GII) confirm the H&E changes and show destructed mitochondria, vacuoles and dilated S.E.R cisternae in some cells. These results are confirmed by Petersen and Soder[53]. Changes in (G II) are coincided with statistical results of significant elevation in MDA and significant decrease in testosterone in comparison to control group.

These are in harmony with Tosca[54] who add that Metformin reduces androgen production by human thecal cells. Metformin affect the gene proteins which is participated in steroid production and therefore decreasing in testosterone production and sperm count[52]. This is supported by the statistical results. These are in harmony with Tosca *et al.*[54]. Who add that MET can reduce GnRH activity at neuron sites and reduces LH and FSH secretion. Affected Leydig cell in this group can be another reason for decreased TH. Statistical results match and show significant decreased in A.R. immunostaining[54]. Also, Rattan *et al.*[55] confirm the impact of Metformin on steroid production in several species (human, rodents, cows

and goats). Interestingly, PCNA activity in (G II) show statically significant decreased staining. This is confirmed by Kayampilly and Menon[34] who explain that by AMPK activation due to MET.

Elevated ROS in (G II) also cause DNA fragmentation and protein degradation leading to cell apoptosis. This finding is supported by significant statically increased caspase -3 immunostaining in this group[56].

CONCLUSION

It is the first research records the direct histological effects of Metformin and Sitagliptin on tests. The results of our study suggest that MET may cause moderate testicular dysfunction while Sitagliptin has severe effects. The risk-benefit ratio of these anti-diabetic drugs requires to be estimated and carefully monitored especially in young male diabetic patients. Further investigations are necessary to clarify the possible long-term differences between both oral anti-diabetic drugs.

CONFILICTS OF INTEREST

There are no conflicts of interest

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الملخص العربى

دراسة هستولوجية وهستوكيميائية مناعية مقارنة لتأثير أدويه السكر (ميتفورمين مقابل سيتاجليبتين) على خصي الفار الابيض البالغ

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مقدمة: مرض السكري مرض خطير له مضاعفات كثيره على اعضاء الجسم المختلفة ومع ذلك قليل من الأدوية المستخدمة في علاج هذا المرض تم دراسة تأثير مضاعفاتها على الخصيه في الجهاز التناسلي للذكور.

الغرض من البحث: مقارنه تأثير عقاري الميتفورمين والسيتاجليبتين كثيري الاستخدام في علاج مرض السكري علي خصي ذكور الفئران البيضاء البالغة من خلال دراسة هستولوجيه وهستوكيميائيه مناعية.

طريقه البحث: استخدم في هذا البحث عدد 50 من ذكور الفئران البيضاء البالغة وقسمت الي ثلاث مجموعات، المجموعة الاولي استخدمت كضابط (ج1) المجموعة الثانية أعطيت عقار الميتفورمين (ج2) المجموعة الثالثة أعطيت عقار سيتاجليبتين (ج3) وفي نهاية البحث استخلصت الخصي ومررت لعمل شرائح هستولوجيه وصبغت بصبغات هيماتوكسيلين و ايوسين و مالوري ترايكروم كما صبغت بصبغات كاسباز و بيكنا و مجسات هرمونات الذكورة الهيستوكيميائيه و جهزت شرائح للفحص بالميكروسكوب الإلكتروني.

النتائج: اظهرت خصي الفئران التي تناولت عقار السيتاجليبتين تغيرات هيستولوجيه كبيره متمثلة في فقد للتركيب الطبيعي لكثير من الأنابيب المنوية في الخصيه ونقص وفقد كبير في الخلايا المنوية وخلايا سيرتولي المبطنة للأنابيب مع موت لكثير من الخلايا وظهور تجاويف للسيتوبلازم في الخلايا وكذلك تأثرت خلايا ليديج الموجودة في النسيج الضام بين الأنابيب كما لوحظ نقص كبير في عدد الحيوانات المنوية داخل الانابيب كما اظهرت دراسة الميكروسكوب الالكتروني تأثيرات شديدة على كل انواع الخلايا النطفية. وكذلك ترسيب قوي للكولاجين بصبغة ماثون تراي كروم. كما ظهر تفاعل قوي مع صبغه الكاسباز وبيكنا ومجسات هرمونات الذكورة له دلالة ملحوظة احصائية. وتناسب ذلك مع تحليل الكمي لكل من قياس مستوي هرمون التستسترون وعدد وحركة الحيوانات المنوية وقطر انابيب النطفية وارتفاع الخلايا النطفية المبطنة لها وعدد خلايا سورتولي التي سجلت كلها انخفاض له دلالة ملحوظة احصائيا بينما سجلت نسبة (MDA) ارتفاع له دلالة ملحوظة إحصائية. أما الفئران التي تناولت عقار الميتفورمين فقد اظهرت تغيرات بسيطة الي حد ما في التركيب الهستولوجي حيث ظهرت معظم الأنابيب بشكلها الطبيعي ومبطنه بالخلايا مع ظهور بعض التجاويف في سيتوبلازم بعض الخلايا المبطنة للأنابيب ولوحظ نقص بسيط في عدد الحيوانات المنوية كما اظهرت دراسة الميكروسكوب الالكتروني تغييرات متفقة مع باقي الصبغات. كذلك ترسيب متوسط لألياف الكولاجين مع صبغة ماثون تراي كروم. ظهر تفاعل متوسط مع صبغه الكاسباز وبيكنا ومجسات هرمونات الذكورة له دلالة ملحوظة احصائية. وتناسب ذلك مع تحليل الكمي لكل من قياس مستوي هرمون التستوستيرون وعدد الحيوانات المنوية التي سجلت انخفاض له دلالة ملحوظة احصائيا بينما سجلت نسبة (MDA) ارتفاع له دلالة ملحوظة إحصائية.

الاستنتاج: كل من العقارين له تأثير على خصيه الفار الابيض ولكن عقار السيتاجلييتين له تأثير أكثر تدميرا على الخصيه لذلك ينصح بعدم تناول هذا العقار لذكور مرضي السكري صغار السن واستخدام عقار الميتفورمين مع المتابعة والفحوص المستمرة. حيث ان استخدام السيتاجلييتين على المدي الطويل يمكن ان يؤدي الي العقم كما ينصح بعمل مزيد من الدراسات لكل من العقارين.