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Impaired expression of sex hormone receptors in male reproductive organs of diabetic rat in response to oral antidiabetic drugs

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Abstract

Introduction. Few oral antidiabetic drugs have been evaluated for their reproductive complication. This study aimed to evaluate the effect of metformin, pioglitazone and sitagliptin on the structure of male reproductive system through an immunohistopathological study.

Material and methods. Sprague-Dawley male rats were injected with streptozotocin. The diabetic rats were divided into four groups (n = 8/each group); diabetic control, metformin-, pioglitazone- and sitagliptin-treated groups in addition to a normal control group (n = 8). At the end of the experiment, blood samples were collected for biochemical assessment. Testis, epididymis and seminal vesicle were dissected and processed for histopathological examination using routine and immune-staining.

Results. All drugs significantly ($p < 0.05$) decreased fasting blood glucose, glycated hemoglobin, total cholesterol, triglycerides and malondialdehyde compared to the diabetic control group. Metformin has induced the least pathologic changes on the structure of the testis, epididymis and seminal vesicle among the studied drugs. Metformin succeeded to restore weights of testis, epididymis and seminal vesicle as well as testosterone hormone level back to values of the NC group while the pioglitazone and sitagliptin failed to do that. A significant reduction ($p < 0.05$) in testicular ERα and ERβ immunoexpression of pioglitazone-treated group as well as suppression of ERβ and AR immunoreactivity in in epididymus and seminal vesicles of pioglitazone- and sitagliptin-treated rats were observed compared to the control animals.

Conclusions. Histological structure as well ER and AR expression in the system organs were negatively and significantly affected with all studied drugs. Metformin has the least effect on the structure of the studied male reproductive organs. Thus, pioglitazone and sitagliptin treatment should be avoided in young male diabetic patients. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 1, 35–48)*

Key words: streptozotocin; male reproductive tract; pioglitazone; sitagliptin; metformin; hormone receptors; IHC

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized mainly by hyperglycemia and leading to complications in many integral body organs including reproductive system [1, 2]. The reported testicular changes of streptozotocin (STZ)-induced diabetic rats are not caused by a direct effect of STZ, but by diabetes itself [3]. Therefore, STZ-induced diabetes in rats is used as a model for studying effects of diabetes on various body organs [4, 5]. A closer look into fertility rates of modern societies reveals that the increased incidence of DM has been closely associated with falling birth rates and fertility [6, 7]. This is due to a disturbing increase of diabetic men

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in reproductive age as well as the multiple molecular mechanisms and pathways are affected by DM with dramatic consequences to male reproductive function [8]. Several reports also demonstrated that DM is associated with hormonal deregulation, particularly of sex steroids hormones [9, 10].

Metformin (dimethylbiguanide) is a drug that reduces blood glucose level and improves the glucose tolerance without altering the plasma insulin profile [11, 12]. It has been used clinically for over 50 years and is recognized as the first-line drug of choice for the treatment of patients with type 2 diabetes (T2D) [13].

Sitagliptin was introduced into the diabetes therapeutic armamentarium in 2006. Since that date the use of dipeptidyl peptidase-4 (DPP-4) inhibitors for the management of type 2 diabetic patients has increased. Continued assessment of adverse events of sitagliptin reported from clinical trials and post--marketing environment is ongoing [14]. Pioglitazone (a thiazolidinedione) acts as an insulin sensitizer and is used for treating type 2 diabetes [15]. It improves insulin sensitivity; impair glucose tolerance and dyslipidemia [16].

Androgens are steroid hormones primarily involved in the establishment of sexual maturation at puberty, and in maintenance of the male reproductive function, spermatogenesis and sexual behavior during adult life [17]. It is well established that androgen receptors (AR) are expressed widely throughout the developing male reproductive system [18]. Many studies have proved that not only androgens have important functions in the adult male reproductive tract, but also estrogen and its receptors are "essential" for normal fertility in the male [19–20].

Only few antidiabetic drugs have been evaluated for their reproductive complications [21]. Therefore, this study was designed to evaluate effects of the commonly used oral antidiabetics; metformin, pioglitazone and sitagliptin; on the male reproductive system (testis, epididymis and seminal vesicle) in STZ-induced diabetic rats through immunohistopathological study. The study used both androgen and estrogen receptors as indicators of the integrity of the male reproductive system.

Material and methods

Animals. This experimental study was approved by the King Abdulaziz University Research Ethics Committee (KAU--REC) and adhered to the international guidelines for use of experimental animals. Sprague-Dawley male rats aged three months and weighing 250–300 g were recruited from King Fahd Research Center, KAU, SA, were acclimated for 6 days before use and were housed in plastic cages in an air-conditioned room at 24°C in a 12 h light–dark cycle with food and water available *ad libitum* [22].

Induction of diabetes mellitus and administration of drugs. Thirty five rats were injected intraperitoneally (ip) with low dose of STZ (35 mg/kg) as was described by Srinivasan et al. [23]. In one week after STZ injection, blood was drawn from the tail vein and blood glucose was measured. Rats with 16.65 mmol/L (300 mg/dL) non-fasting blood glucose level were considered to be diabetic rats (n = 32). They were randomly allocated to four groups (n = 8): diabetic control (DC) group that received no treatment and three drug-treated groups receiving metformin at the dose 100 mg/kg/day) according to Sun et al. [24], pioglitazone (20 mg/kg/day) according to Ding et al. [15] and sitagliptin (30 mg/kg/day) according to Chen et al. [25]. All drugs and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Drugs were dissolved in distilled water and given in a volume of 1 mL for 6 weeks by oral gavage. Five rats designated as normal control (NC) group were given vehicle citrate buffer (pH 4.4) in a volume of 1 mL/kg, ip and were fed standard chow [23].

Measurements of body weight (BW) and blood parameters. Rats' body weight was measured initially, weekly and at the end of the study. A blood sample (3 mL) was collected after an overnight fast at the end of the study from each rat by capillary tubes *via* the retroorbital sinus under light anesthesia by ether. Each sample was divided into two parts. The first part (2.5 mL) was placed in a plain tube for separation of serum which was stored at −80°C until measurement of the levels of fasting blood glucose (FBS), total cholesterol (TC), triglycerides (TG), malondialdehyde (MDA) and testosterone by using commercially available colorimetric kits. The latter was determined in duplicate using the Testosterone Enzyme Immunoassay kit (Assay Design Inc., Ann Arbour, USA) according to the manufacturer's instructions. The detection limit for this assay was 3.82 pg/mL; cross reactivity with corticosteroid and other androgens was minimal (< 1%). Serum insulin level was measured by using a rat insulin ELISA assay kit. The second part (0.5 mL) was placed in an EDTA tube for measurement of glycated hemoglobin (HbA_{1c}). All kits were purchased from Biocompare (South San Francisco, CA, USA) and measurements were done in accordance with the manufacturer's protocol [22, 26].

Tissue sampling. Animals were anesthetized with diethyl ether and sacrificed by cervical dislocation then perfused with 10% neutral-buffered formalin. Testis, epididymis and seminal vesicle were dissected out, weighted, trimmed, fixed in Bouin overnight then processed to obtain paraffin blocks. Serial paraffin sections were cut into 5 μm thickness and stained with hematoxylin and eosin (H & E) and Masson trichrome (MT) according to Drury and Wallington [27].

Immunohistochemical methods. Paraffin sections were immunostained using an avidin–biotin technique according to Nie et al. [28]. Paraffin sections on positive slides were immunostained using an avidin–biotin technique. Slides were deparaffinized, rehydrated, rinsed in tap water, and embedded in 3% H_2O for 10 min to block endogenous peroxidase. Sections were immersed in an antigen retrieval solution (10 mmol/L sodium citrate buffer, pH 6) and subjected to heat-induced antigen retrieval for 20 min in a microwave. Nonspecific protein binding was blocked by blocking solution (PBS + 10% normal goat serum). The slides were incubated for 30 min with the diluted primary antibodies. Estrogen receptor alpha (ERα) was immunolocalized in the tissue using a mouse monoclonal antibody (NCL-ER-6F11), which is raised to the N-terminal (A/B) region of the human ERα at a 1:1,000 dilution according to that of Attia and Elmansy [29]. The monoclonal antibodies and diaminobenzidine were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Estrogen receptor beta (ERβ) was localized using rabbit polyclonal antipeptide IgGs raised against a specific peptide located in the D region of rat ERβ (Dako, Glostrup, Denmark) at a dilution 1:100 to 1:250 as described in Saunders et al. [30]. Androgen receptor (AR) was localized using rabbit anti-AR (N-20, Santa Cruz Biotechnology, CA, USA), which was diluted 1:250 in phosphate-buffered saline and 0.25% bovine serum albumin and maintained at room temperature overnight. Following primary antibody incubation, sections were incubated with goat anti-rabbit biotinylated secondary antibody followed by an avidin–biotin horseradish peroxidase complex (Dako). Drops of streptavidin peroxidase were added to the slide, left for 20 min, and then washed with PBS for 5 min. Diaminobenzidine was added to slides as a chromogen, after which the slides were washed with distilled water. Finally, the slides were stained with Mayer's hematoxylin, dehydrated, and cleared in xylene. For the negative control slides, the specific primary antibody was replaced by PBS. Monoclonal mouse anti-antibody kits and diaminobenzidine were obtained from Sigma-Aldrich. Sections incubated without the primary antibody but with PBS were used as negative controls for color development on the same slide. All positive reactivity was noted as predominantly nuclear and cytoplasmic brown staining.

An Olympus Microscope BX-51 with a digital camera connected to a computer was used for photographing. The Pro Plus image analysis software version 6.0 was used for the measurement of area percentage of ERα, ERβ and AR immunoexpression in the testis, epididymis and seminal vesicle of the immunohistochemically stained sections. The mean cross-sectional area and the mean germinal epithelium height of the seminiferous tubules (STs) were measured. Five sections were analyzed for each animal. Five readings from each section were measured and the mean for each animal was calculated according to Zhou et al. [31]. The morphometric data was presented as per Editor recommendation.

Statistical analysis. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 16. For non-parametric data, Analysis of Variance (ANOVA) Kruskal-Wallis followed by a *post-hoc* test (based on the Dunn procedure) was used to analyze each pair of groups, thereby avoiding a multiple-comparison effect. For the parametric data, the different groups were compared using ANOVA (f test), followed by a Bonferroni *post hoc* test. A p value less than 0.05 was considered to be significant.

Results

Biochemical findings and body weight changes

There were no significant differences in glucose level among the groups at start of the study (data not shown). All groups showed significant higher FBS and HbA_{1c} compared to that of the NC rats. All drugs significantly decreased the FBS and HbA_{1c} compared to the DC rats minimally with sitagliptin (Table 1). All drugs significantly increased serum insulin compared to DC group maximally with metformin (Table 1). TC, TG and MDA were significantly higher in all groups compared to the NC rats while these parameters were significantly lower in the studied drugs compared to the DC rats minimally with sitagliptin (Table 1). Although testosterone level was significantly reduced in the DC compared to the NC group, it increased in the metformin-treated group decreased in both pioglitazone and sitagliptin-treated groups compared to the DC (Table 1).

At the end of the study all rat groups showed significantly increased body weight (BW) compared to the NC rats and only pioglitazone significantly increased BW compared to the DC rats (Table 1). Although the weights of the testis, epididymis and seminal vesicles were significantly reduced the DC compared to the NC group, these weight significantly increased in all treated groups compared to the DC. In the metformin-treated group the organs' weights showed insignificant difference from the NC group (Table 2).

Histological and immunohistochemical findings

Testis

The testis of the DC rats showed irregular seminiferous tubules (STs) lined with disorganized germinal epithelium and the intervening interstitial connective tissue (ICT) appeared edematous compared to the NC rats (Figure 1A, B). The STs of metformin-treated rats were line with more or less intact germinal epithelium with few deformed spermatocytes with dark nuclei and the ICT was edematous. The same

Table 1. Concentration of selected substances and hormones in blood and serum

	NC	STZ	STZ + metformin	STZ + pioglitazone	STZ + sitagliptin
FBG [mmol/L]	5.73 ± 0.6	20.81 ± 0.9[a]	12.94 ± 0.7[a, b]	13.25 ± 2.1[a, b]	15.79 ± 1.8[a, b]
HbA_{1c} (%)	4.45 ± 0.3	11.61 ± 0.9[a]	7.05 ± 0.9[a,b]	7.45 ± 0.7[a ,b]	10.35 ± 0.7[a, b]
Insulin [pmol/L]	266.90 ± 14.1	241.50 ± 11.5[a]	287.60 ± 6.8[a, b]	259.40 ± 6.5[a, b]	256.90 ± 8.8[a, b]
Cholesterol [mmol/L]	1.36 ± 0.2	5.09 ± 0.5[a]	3.15 ± 0.4[a, b]	3.88 ± 0.7[a, b]	4.05 ± 0.5[a, b]
TG [mmol/L]	0.47 ± 0.1	1.97 ± 0.5[a]	1.39 ± 0.3[a, b]	1.49 ± 0.2[a, b]	1.52 ± 0.2[a, b]
MDA [nmol/mL]	1.64 ± 0.2	11.93 ± 0.5[a]	5.20 ± 0.5[a, b]	7.11 ± 0.7[a, b]	10.74 ± 0.9[a, b]
Testosterone [ng/mL]	1.4 ± 0.1	000.70 ± 0.04[a]	1.13 ± 0.09[a, b]	0.21 ± 0.2[a, b]	0.21 ± 0.05[a, b]

Data express mean ± standard deviation. [a]significantly different from normal control rats (NC); [b]significantly different from diabetic rats (STZ), $p < 0.05$. Abbreviations: NC — normal control group; STZ — rats treated with streptozotocin; FBG — fasting blood glucose; HbA_{1c} — glycated hemoglobin; TG — triglycerides; MDA — malondialdehyde

Table 2. Body weight and weight of male reproductive organs of the studied rat groups

	Groups				
	NC	STZ	STZ + metformin	STZ + pioglitazone	STZ + sitagliptin
Body weight [g]	258.40 ± 14.9	280.00 ± 12.3[a]	274.10 ± 7.1[a]	301.80 ± 12.2[a, b]	269.90 ± 5.1[b]
Right testis weight [g]	1.5 ± 0.09	0.58 ± 0.10[a]	1.32 ± 0.05[b]	1.2 ± 0.04[a, b]	1.14 ± 0.03[a, b]
Left testis weight [g]	1.7 ± 0.05	0.60 ± 0.09[a]	1.42 ± 0.06[b]	1.20 ± 0.44[a, b]	1.19 ± 0.20[a, b]
Right epididymis weight [g]	0.36 ± 0.06	0.24 ± 0.01[a]	0.31 ± 0.03[b]	0.21 ± 0.02[a, b]	0.18 ± 0.01[a, b]
Left epididymis weight [g]	0.32 ± 0.01	0.25 ± 0.02[a]	0.32 ± 0.02[b]	0.22 ± 0.01[a, b]	0.17 ± 0.01[a, b]
Right seminal vesicle weight [g]	0.44 ± 0.05	0.23 ± 0.02[a]	0.38 ± 0.08[b]	0.26 ± 0.07[a]	0.25 ± 0.02[a]
Left seminal vesicle weight [g]	0.45 ± 0.06	0.21 ± 0.05[a]	0.39 ± 0.08[b]	0.24 ± 0.07[a]	0.23 ± 0.05

Abbreviations as for Table 1. Data express mean ± standard deviation. [a]significantly different from normal control rats (NC); [b]significantly different from diabetic rats (STZ), $p < 0.05$

changes were observed in the less affected STs of pioglitazone-treated rats while the markedly affected tubules were atrophied. Testis of sitagliptin-treated rats showed STs with most of the spermatocytes in different mitotic stages (Figure 1C, E). There was significant reduction in the mean cross-sectional area of the SNTs and the germinal epithelium height of both pioglitazone- and sitagliptin-treated groups while the metformin-treated group showed insignificant reduction compared to the NC (Table 3). Although the testicular tissue of DC, metformin-, pioglitazone-, sitagliptin-treated groups showed an increase in the amount of collagen fibers, the area percent of the MT -stained collagen fibers showed insignificant increase compared to the NC group (Figure 1F–K).

Regards the immunolocalization of estrogen and androgen receptors in testicular tissue, the NC group showed immunoexpression of ERα in the Leydig cells (LC) that was reduced in the untreated diabetic group as well metformin-, pioglitazone- and sitagliptin-treated groups. This reduction was statistically significant only in untreated diabetic and pioglitazone-treated groups compared to the NC (Figure 2A–F). Control testis showed ERβ immunoexpression in Sertoli cells (SC), spermatogonia, LC and spermatids. The percent of ERβ-stained area was significantly decreased in the DC and pioglitazone-treated groups when compared to the NC (Figure 2G–L). Control group showed AR immunoexpression in SC, peritubular myoid cells, LC and the spermatids (Figure 2M–R).

Epididymis

Corpus of the control epididymis was lined with pseudostratified columnar epithelium with stereocilia and surrounded by thin peritubular smooth muscle layer. Numerous principal cells, basal cells, less apical cells and narrow cells were seen among the lining epithelium. Cauda of the control epididymis appeared with wider lumen and surrounded by thicker peritubular muscle layer (Figure 3A, B). Both epididymal corpus and cauda of the DC, metformin-, pioglitazone- and sitagliptin-treated groups showed

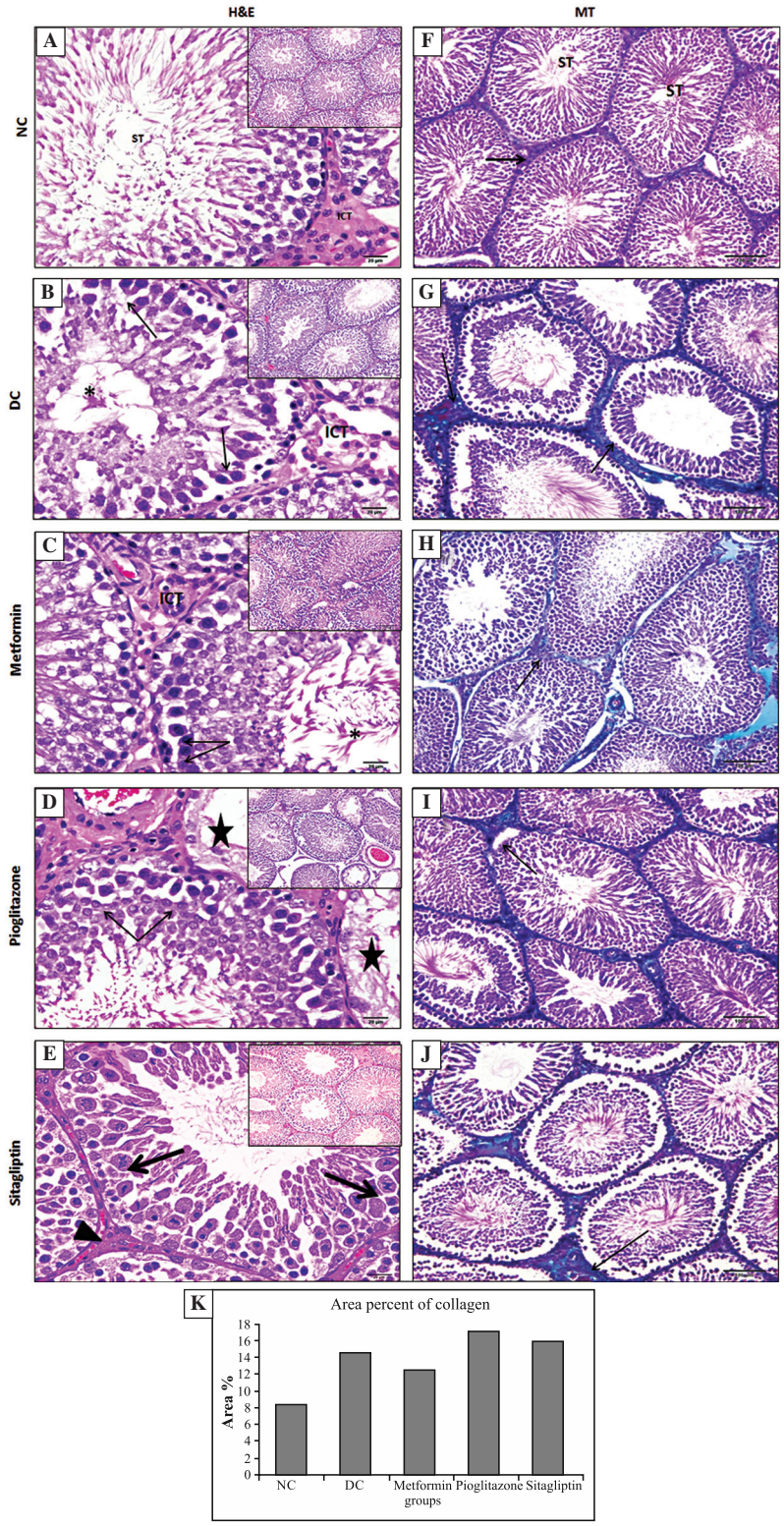

Figure 1. A. Normal control rat has intact structure of testis; **B.** Testis of diabetic rat; **C.** Testis of diabetic rat treated with metformin show deformed primary spermatocytes (arrow) with dark nuclei, few spermatozoa in the lumen (asterisk) and edematous interstitial connective tissue with some cellular vacuolization; **D.** Testis of diabetic rat treated with pioglitazone shows atrophied STs (star) while another ST shows deformed spermatocytes with dark nuclei (arrow); **E.** Testis of diabetic rat treated with sitagliptin shows reduced germinal epithelium, many spermatocytes in the mitotic stages (thick arrow) and congested capillaries (arrow head) (H & E staining: × 600, insert: × 200); **F.** Normal control rat testis shows minimal collagen fibers (arrow); **G.** Testis of diabetic rat; **H.** Testis of diabetic rat treated with metformin or pioglitazone (**I**), or sitagliptin (**J**) show excess collagen fibers (arrow) (Masson trichrome staining: × 200); **K.** The area percent of the collagen fibers all groups. Abbreviations: NC — normal control rats; DC — diabetic control rats (STZ-induced diabetes); H & E — hematoxylin and eosin; ST — seminiferous tubule

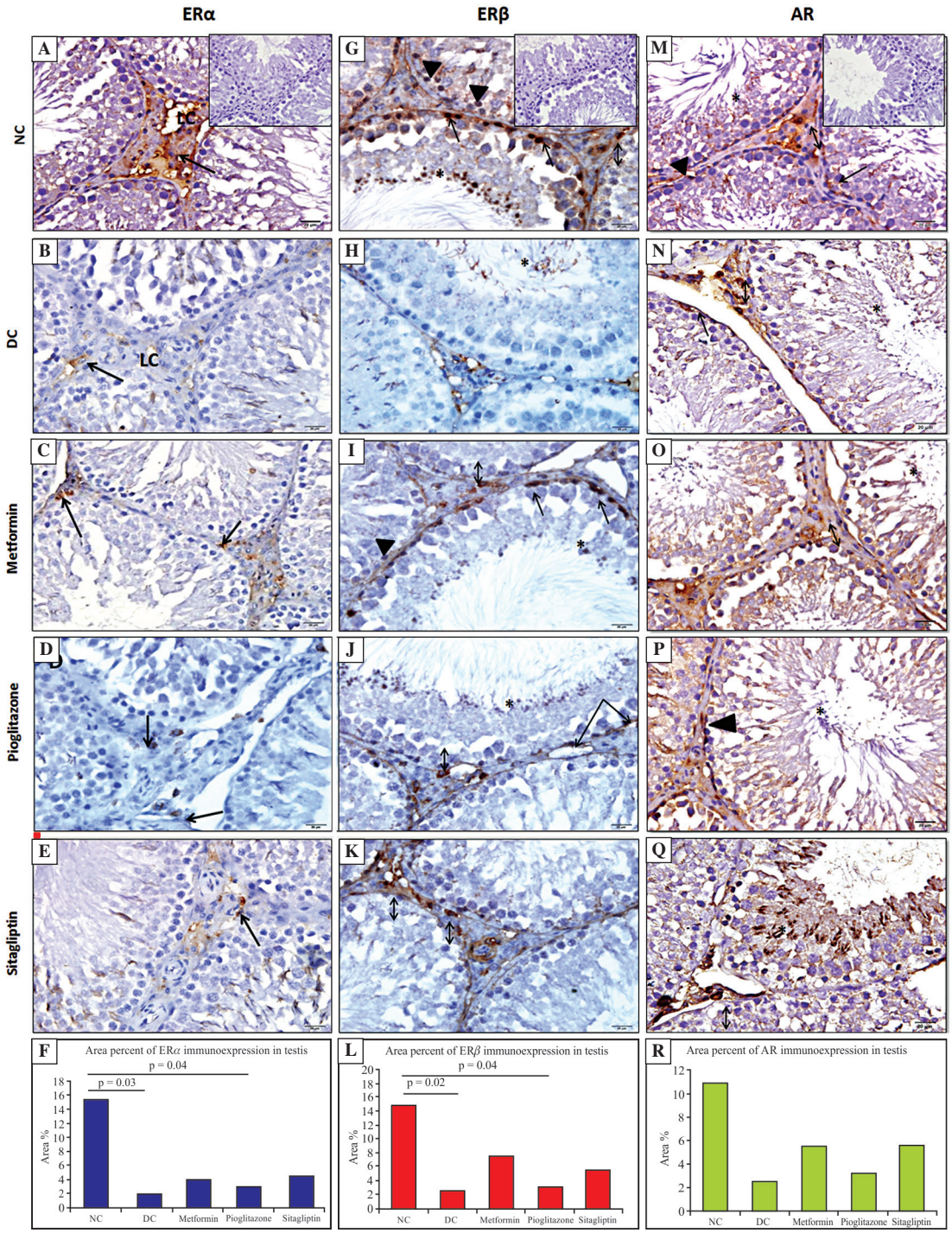

Figure 2. A. Testis of normal control rat shows ERα expression in the interstitial LC; **B**. Testis of diabetic rat and testis of diabetic rat treated with metformin (**C**), pioglitazone (**D**) and sitagliptin (**E**) show markedly reduction (arrow) in ERα expression (anti-ERα immunostaining: × 600); **F**. The area percentage of ERα immunoexpression in testis of all groups; **G**. Testis of normal control rat shows ERβ expression in SC (arrow), spermatogonia (arrow head), LC (bi-head arrow) and round or elongated spermatid (asterisk); **H.** Testis of diabetic rat; **I.** Testis of diabetic rat treated with metformin or pioglitazone (**J**), or sitagliptin (**K**) show reduction in ERβ expression (ERβ immunostaining: × 600); **L.** The area percentage of ERβ immunoexpression in testis of all rat groups; **M.** Testis of normal control rat shows AR expression in SC (arrow), PMC (arrowhead), LC (bi-head arrow) and round or elongated spermatid (asterisk); **N.** Testis of diabetic rat; **O.** Testis of diabetic rat treated with metformin or pioglitazone (**P**), or sitagliptin (**Q**) show reduction in AR immunoexpression (anti-AR immunostaining: × 600); **R.** The area percentage of AR expression in the testis of all groups.
Note. Inserts show negative control slides. Abbreviations: ERα — estrogen receptor α; LC — Leydig cells; ERβ — estrogen receptor β; SC — Sertoli cells; AR — androgen receptors; PMC — peritubular myoid cells

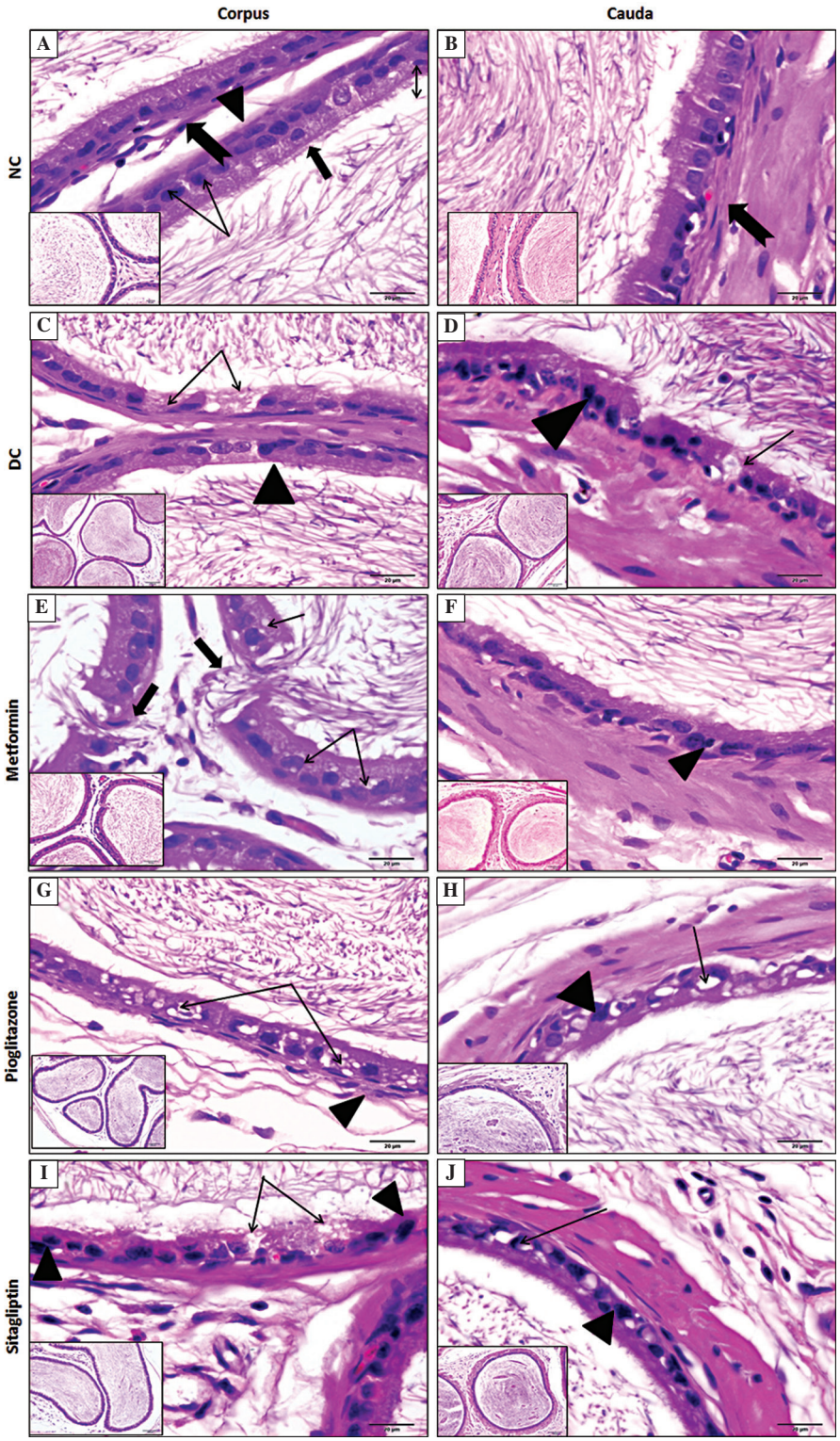

Figure 3. A. Corpus of epididymis of normal control rat is lined by pseudostratified columnar epithelium with stereocilia and numerous principal cells (thick arrows), basal cells (arrow head), less apical cells (thick arrow), narrow cells (bi-head arrow) and thin PSM (bifid arrow); **B**. Cauda of the normal control rat shows wider lumen and thicker PSM (bifid arrow); **C.** Corpus and cauda **(D)** of epididymis of diabetic rat; **E.** Corpus and cauda **(F)** of epididymis of diabetic rat treated with metformin show clumping of the nuclei of epithelial cells, cytoplasmic vacuoles (arrow) and disruption of the epididymal duct wall (thick arrow) with the leakage of sperms; **G.** Corpus and cauda **(H)** of epididymis of diabetic rat treated with pioglitazone; **I.** Corpus and cauda **(J)** of diabetic rat treated with sitagliptin show epithelial cells with many cytoplasmic vacuoles (thin arrow) and dark nuclei (arrowhead) (H & E staining: × 1,000, insert: × 200). Abbreviations: as for Figure 1

Table 3. Morphometric measurements in male reproductive organs of the studied groups

Parameter	Groups				
	NC	STZ	STZ + metformin	STZ + pioglitazone	STZ + sitagliptin
Testis: cross-sectional area of seminiferous tubules [$\times 10^2$ μm^2]	144.3 ± 10.6	316.8 ± 15.7[a]	177 ± 50.3[b]	309 ± 61.2	384.3 ± 28[b]
Testis: germinal epithelium height [μm]	35.2 ± 2.7	23.1 ± 4.9[a]	33.9 ± 5.2[b]	20.5 ± 11.9	20.3 ± 1.9
Corpus of epididymis: epithelium height [μm]	23.3 ± 0.71	12.8 ± 0.72[a]	21 ± 1.9[b]	14.3 ± 0.7[b]	16.9 ± 1.3[b]
Cauda of epididymis: epithelium height [μm]	20.6 ± 1.4	14.1 ± 1.2[a]	16.3 ± 2.4[b]	13.1 ± 0.7	13.5 ± 1.5

Abbreviations as for Table 1. Data express mean ± standard deviation. [a]significantly different from normal control rats (NC); [b]significantly different from diabetic rats (STZ), $p < 0.05$

some epithelial cells with dark nuclei while other cells showed cytoplasmic vacuoles. The DC and metformin -treated groups showed clumping of the nuclei of the epithelial lining. The metformin-treated group showed disruption of many ducts of the epididymal corpus with leakage of sperms outside the duct (Figure 3C–J). There was significant reduction in the epithelial cell height of both corpus and cauda of epididymis of all treated groups compared to the NC (Table 3).

Corpus and cauda of the control epididymis showed strong to moderate ERβ immunoexpression in the principal cells of the lining epithelium, sperm, interstitial cells and the peritubular smooth muscles (Figure 4A, B). The DC group showed moderate to weak ERβ expression. Metformin-, pioglitazone- and sitagliptin-treated groups showed marked decrease in ERβ expression in the epithelium and peritubular muscle while the interstitial cells and sperm still showed positive ERβ expression. There was significant decrease in the area percent of ERβ expression in both corpus and cauda of the epididymis of the DC group as well as pioglitazone- and sitagliptin-treated groups compared to the NC (Figure 4C–L). Corpus and cauda of the control epididymis showed strong nuclear AR expression in the lining epithelium, sperm and interstitial cells while the peri-tubular smooth muscles appeared negative (Figure 5A, B). The DC group as well as all the treated groups showed moderate to weak cytoplasmic AR expression in epithelial cells of corpus and cauda while the interstitial cells still show positive expression. Statistical analysis showed significant decrease in the area percent of AR expression in the corpus of the DC and pioglitazone- and sitagliptin-treated groups and significant decrease in the cauda of the DC compared to the NC (Figure 5C–L).

Seminal vesicles

Seminal vesicle of the DC group showed disorganized epithelium and increased intraepithelial lymphocytes compared to the NC (Figure 6A, B). Similar changes were observed in metformin-, pioglitazone- and sitagliptin-treated groups with evident degree in sitagliptin-treated group (Figure 6C–E). Control seminal vesicle showed ERβ immunoexpression in the epithelial cells and the smooth muscle fibers (SMF). The DC, pioglitazone and sitagliptin-treated groups group showed week expression in few epithelial cells and negative expression in SMF while metformin-treated group showed few epithelial cells with strong ERβ expression. There was significant decrease in the area percent of ERβ expression of the DC, pioglitazone- and sitagliptin-treated groups compared to the NC (Figure 6F–K). Control seminal vesicle showed moderate AR immunoexpression in the epithelial cells and negative expression in the SMF while the DC group showed negative expression in both epithelium and SMF. Metformin-, pioglitazone- and sitagliptin -treated groups showed week AR immunoexpression in the epithelial cells. There was significant decrease in the area percent of AR expression in the seminal vesicle of the DC, pioglitazone- and sitagliptin-treated groups compared to the NC and significant decrease in pioglitazone- and sitagliptin-treated groups compared to the DC (Figure 6L–Q).

Discussion

Estrogens are essential to preserve the structural and functional integrity of the male reproductive tract and their α-receptors are necessary for male fertility [32]. Structural changes of the reproductive system in DM have been reported in several studies [33–35] while little ones dealt with the effect of the different antidiabetic drugs on the structure of such system. This study aimed to evaluate the effects of the commonly used oral antidiabetics; metformin, pioglitazone and sitagliptin on the structure of male reproductive system in a STZ-induced diabetic rats. It was observed that the therapies studied in this work succeeded in controlling the STZ-induced diabetic

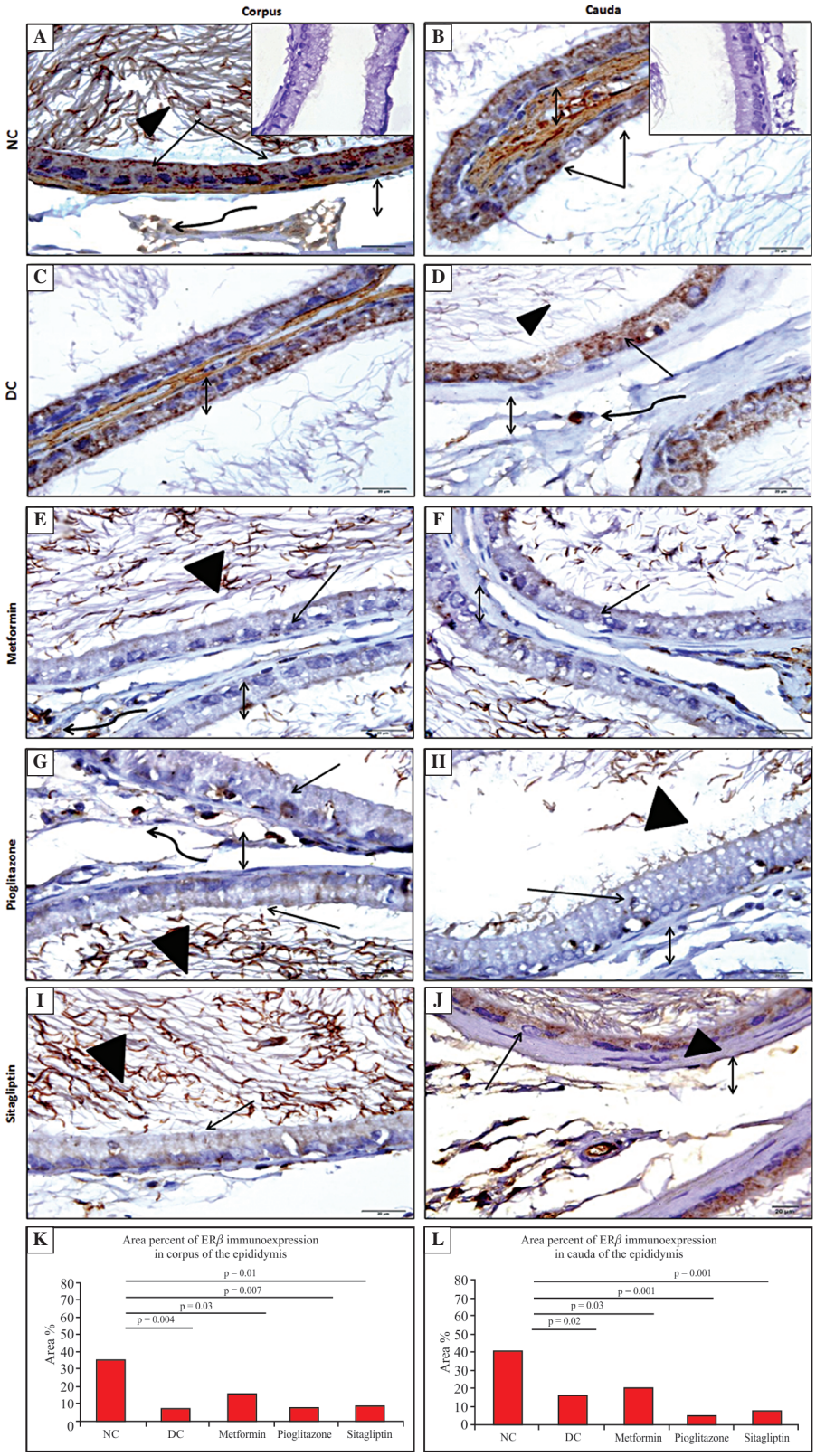

Figure 4. Epididymal corpus (**A**) and cauda (**B**) of normal control rat show strong to moderate ERβ expression mainly in the principal cells (arrows), sperm (arrowhead), interstitial cells (curved arrow) and the PSM (bi-head arrow); **C.** Corpus and cauda (**D**) of diabetic rat show moderate to weak ERβ expression (arrow); **E.** Corpus and cauda (**F**) of diabetic rat treated with metformin or pioglitazone (**G** and **H,** respectively), or sitagliptin (**I** and **J,** respectively) show decreased ERβ expression in epithelium (arrow) and PSM (bi-head) while interstitial cells and sperm show strong expression (arrow) (anti-ERβ immunostaining: × 1,000); **K, L.** The area percentage of ERβ expression in both corpus and cauda, respectively, in all rat groups. Abbreviations: as for Figure 2

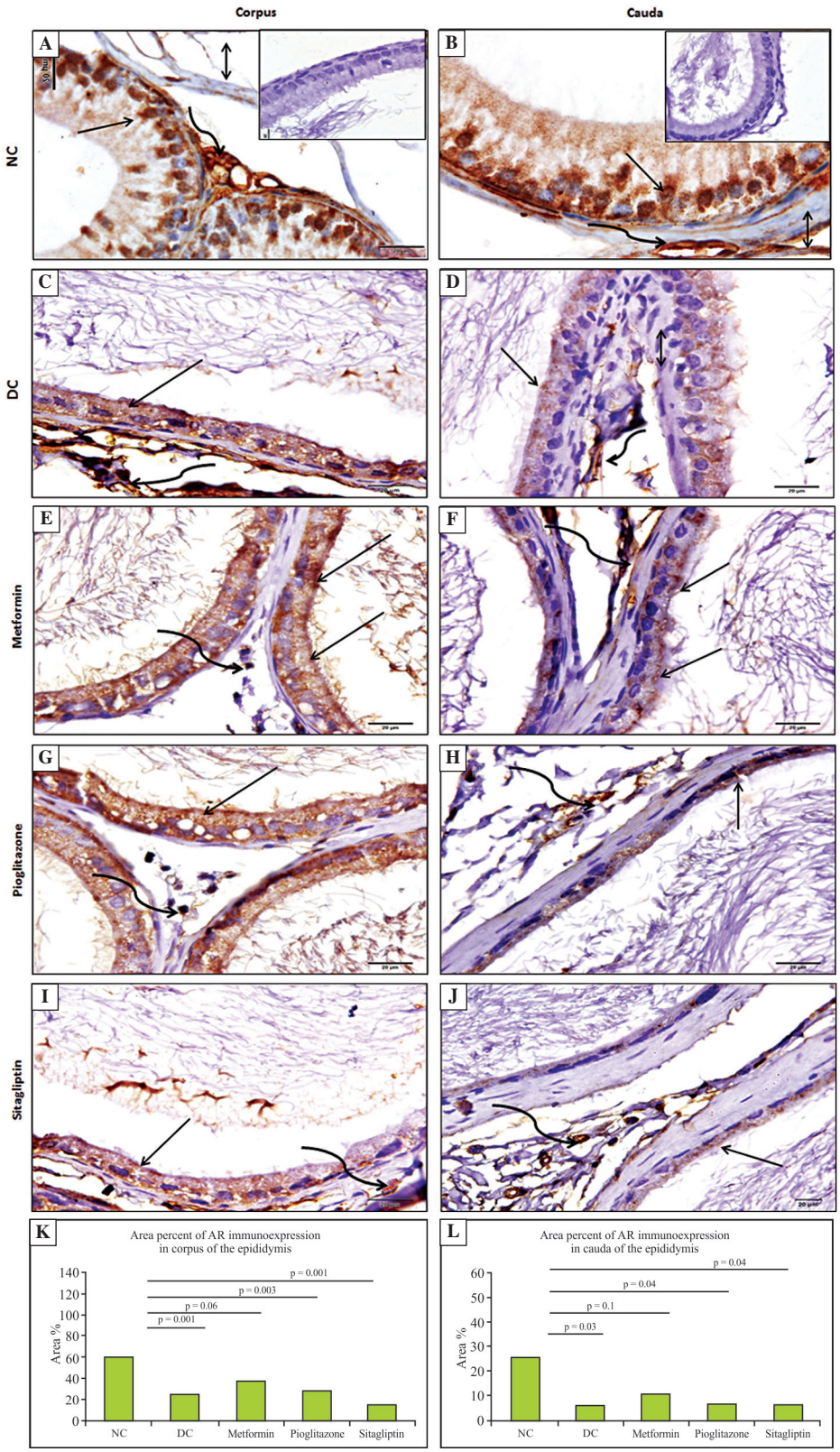

Figure 5. Epididymal corpus **(A)** and cauda **(B)** of the normal control rat show strong AR expression in the nuclei of the lining epithelium (arrow) and interstitial cells (curved arrow), while the PSM (bi-head arrow) appear negative; **C.** Corpus and cauda **(D)** of diabetic rat; **E.** Corpus and cauda **(F)** of diabetic rat treated with metformin or pioglitazone **(G** and **H,** respectively), or sitagliptin **(I** and **J**, respectively) show moderate to weak AR expression (arrow) of corpus and cauda while the interstitial cells (curved arrow) also show positive expression (anti-AR immunostaining: × 1,000); **K.** The area percentage of AR immunoexpression in the corpus and cauda **(L)** of epididymis in all rat groups. *Note.* Inserts show negative control slides. Abbreviations: as for Figure 2

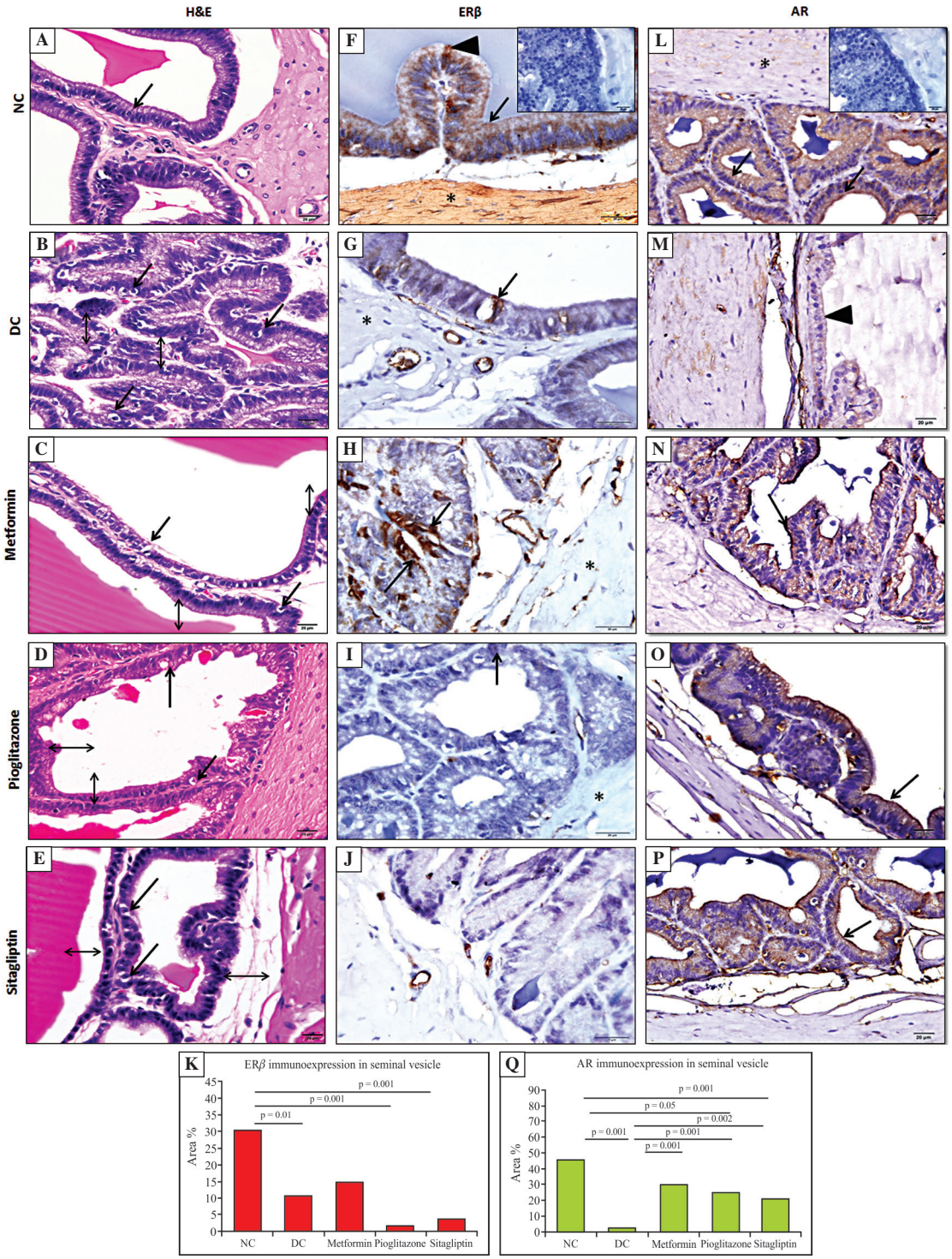

Figure 6. A. Seminal vesicle of normal control rat show intact columnar epithelium (arrow); **B.** Seminal vesicle of diabetic rat or diabetic rat treated with metformin **(C)**, or pioglitazone **(D),** or sitagliptin **(E)** show disorganized epithelium with intraepithelial lymphocytes (arrow) and dark nuclei (bi-head arrow) (H & E: × 600); **F.** Seminal vesicle of normal control rat shows strong to moderate (arrow) and week (asterisk) ERβ expression in epithelial cells and SMF, respectively; **G.** Seminal vesicle of diabetic rat and diabetic rat treated with metformin **(H)** or pioglitazone **(I),** or sitagliptin **(J)** show week expression in few epithelial cells and negative expression in SMF (anti-ERβ immunostaining: × 1,000); **L.** Seminal vesicle of normal control rat shows strong AR expression (arrow) in epithelial cells while SMF show no expression (asterisk); **M**. Seminal vesicle of diabetic rat shows no AR expression in the epithelium (arrowhead); **N.** Seminal vesicle of diabetic rat treated with metformin or pioglitazone **(O),** or sitagliptin **(P)** show moderate expression in the epithelial cells (arrow) (anti-AR immunostaining: × 1,000); **K.** The area percentage of ERβ and AR **(Q)** expression in the seminal vesicle of all rat groups. *Note*. Inserts show negative control slides. Abbreviations: as for Figure 2; SMF — smooth muscle fibers

by reducing hyperglycemia, HbA_{1c}, TC, TGs, MDA, and partially prevented insulinopenia. These results are consistent with the previous studies on metformin [24], pioglitazone [23] and sitagliptin [36, 37].

Although the weights of testis, epididymis and seminal vesicle as well as testosterone hormone level was significantly reduced in the STZ-induced diabetic rats and these findings were consistent with those of previous studies [38–41], metformin succeeded to restore it back to values with insignificant difference compared to the NC group while the pioglitazone and sitagliptin failed to do that. Reduced serum testosterone in diabetic rats was attributed in previous studies to neuroendocrine dysfunction and the absence of the stimulatory effect of insulin on the LC [3, 42].

The disorganized germinal epithelium and edematous thickened interstitial connective tissue in the testis of diabetic rats have been described in the previous studies [33, 40, 42–44] and have been also observed in this study. The crowded nuclei and cytoplasmic vacuoles of the epithelial lining the epididymis of the DC observed in this study was in agreement with those of Soudamani et al. [45] who attributed them to shrinkage of epididymal tubules.

Among the main findings in this study that metformin has induced the least pathologic changes on the structure of the testis and this is in line with the finding of Kianifard et al. [46] and is supported by the recent results of Alves et al. [13] which suggest that metformin may improve the male reproductive potential and it is, therefore, a suitable anti-diabetic agent for young adults and adolescents with T2D, as it will not severely compromise their reproductive function. On the other hand, these results were in contradict with those of Adaramoye et al. [47] who reported marked histological alterations concomitant with a decrease in sperm counts and motility in healthy adult male rats, which were administered metformin, for three consecutive weeks. These reproductive deleterious results could be attributed to having available lower levels of glucose due to the oral antidiabetic metformin which invalidate their results obtained. Pioglitazone- and sitagliptin-treated rats showed markedly histopathologic changes in testis, epididymis and seminal vesicle compared to those treated with metformin and unfortunately, no studies dealt with their effect on the structure of the testis were available to be compared with these results. When it came to the mechanism by which the drugs induced these changes, lipid peroxidation has been suggested as one of the molecular mechanisms involved in drug-induced tissue injuries [48]. In the present study, increased levels of MDA, an index of lipid peroxidation, were observed in the serum of all treated rats however metformin remains the least drug caused this effect. This may be due in part to radical species generated during metabolism of drugs which attack cell membrane phospholipids and other circulating lipids.

Testicular immunoexpression of ERα [28, 29, 49], ERβ and AR [50, 51] as well as seminal vesicle ERβ and AR immunoexpression [30, 52] and epididymal ERβ [31, 53] immunoexpression were all previously described and are consistent with what described in this study. A significant reduction in testicular ERβ and ERβ expression in pioglitazone-treated group as well as in epididymal and seminal vesicle ERβ and AR expression in pioglitazone- and sitagliptin-treated groups were observed compared to the NC. These findings indicate that metformin has the least effect, among the studied drugs, on the estrogens and androgens receptors. It was observed that the DC as well as all the treated groups showed cytoplasmic instead of nuclear AR expression. This finding could be explained in the light of the previous studies reported that, in the presence of androgens, AR is normally localized in the nucleus while in the absence of androgens the receptor migrates from the nucleus back into the cytoplasm [54–56]. Therefore, alterations in normal AR expression in these cells would be an indicator of the hormonal status of the cells [53].

In conclusion, the present study showed different grades of histopathological changes observed in the male reproductive system of type 2 diabetic rats treated with metformin, pioglitazone and sitagliptin. The histological structure as well estrogen and androgen receptors expression in the system organs were negatively affected with all studied antidiabetic drugs with the maximal impact of pioglitazone then sitagliptin. Metformin had the least negative effect on the structure of the reproductive organs. The results of this experimental study suggest that pioglitazone and sitagliptin should be avoided in the treatment of young male diabetic patients.

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