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Angiotensin blockade attenuates diabetic nephropathy in hypogonadal adult male rats

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Abstract

This study examined the effect of the aromatase inhibitor letrozole (0.5 mg/kg) alone or in combination with the angiotensin-receptor blocker valsartan (30 mg/kg) against streptozocin-induced diabetic nephropathy (DN) in hypogonadal (HG) rats for 12 weeks. First, we tested the HG effect on hormone levels, inflammatory cytokines, and oxidative stress in non-diabetic (ND) and diabetic (D) rats. HG was induced with the luteinizing hormone-releasing hormone antagonist cetrorelix (0.71 mg/kg). Diabetes enhanced hormonal hypogonadism and increased inflammation and oxidative stress. Next, experiments examined the effect of early letrozole and valsartan intervention on DN in HG rats. HG-ND and HG-D rats were treated with letrozole alone or in combination with valsartan. HG-D rats developed proteinuria and had increased blood urea nitrogen and creatinine, and histopathological evidence of renal injury, including glomerular hypertrophy and mesangial expansion. Valsartan alone or in combination with letrozole reduced proteinuria, improved renal functions, and reduced diabetes-induced renal angiotensin II. Both agents ameliorated NF-κB, IL-1β, IL-6, and TNFα levels. The combination decreased SOD, MDA, GPx levels, and prevented glomerular hypertrophy. In HG-D rats, valsartan reduced renal collagen IV and TGF-β1, especially when the testosterone level was corrected by letrozole. Thus, normalizing testosterone and inhibiting renal angiotensin II have a renoprotective effect against DN in HG male rats.

Key words: kidney, hypogonadism, diabetic nephropathy, aromatase inhibitor, valsartan, testosterone, estradiol, oxidative stress, angiotensin II, inflammatory cytokines.
Introduction

Diabetic nephropathy (DN) becomes the major cause of chronic kidney diseases with a huge burden on patients and health systems. Pharmacological treatment could delay, without preventing the progression of DN (Cao and Cooper 2011).

DN is the result of disturbed hemodynamic and non-hemodynamic mechanisms (Carmines 2010; Magee et al. 2009). The non-hemodynamic mechanisms are the result of hyperglycemia-associated metabolic abnormalities (Forbes et al. 2007) and hormonal changes (Manigrasso et al. 2012). Hormonal changes in diabetic male patients reportedly show lower serum testosterone levels than the non-diabetic males (Fukui et al. 2007). Chronic kidney disease patients were also suffering from low testosterone levels (Khurana et al. 2014).

Hyperglycemia-associated metabolic abnormalities could be detected by glycated hemoglobin (HbA1c), which reflects average plasma glucose over the preceding 8–12 weeks and is formed by non-enzymatic attachment of glucose to hemoglobin (Nathan et al. 2007). Hyperglycemia is associated with high levels of free radicals and an increase in lipid peroxidation that causes cellular damage and is directly related to diabetic complications (Erejuwa et al. 2011; Maritim et al. 2003). Diabetes is also associated with an enhanced inflammatory state and increases in nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activity and various inflammatory mediators that play key roles in DN (Anderberg et al. 2015; Brahler et al. 2012).

Male hypogonadism (HG) is a syndrome in which there are low physiological levels of testosterone as a result of testicular disease or dysfunction of the hypothalamic-pituitary axis (Basaria 2014). Diabetes is one of the secondary causes of HG in male patients (Basaria 2014). HG has also been observed in experimental diabetic models (Manigrasso et al. 2012; Prabhu et al. 2012).
Low testosterone level is observed in both type 1 and 2 diabetes (Grossmann et al. 2008). Studies on castrated animals with zero-level testosterone showed that androgen replacement has a renoprotective effect (Prabhu et al. 2010; Sun et al. 2007; Xu et al. 2009; Xu et al. 2008). However, testosterone replacement therapy in patients with or without renal disease is controversial (Bao and Johansen 2015; Raynaud 2009). In both young and aged HG men, interventional and observational studies have revealed that testosterone supplementation can reduce inflammatory markers such as interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and IL-1β (Maggio et al. 2005; Malkin et al. 2004; Manigrasso et al. 2012; Vodo et al. 2013).

Hemodynamic mechanisms are involved in DN pathogenesis through the elevation of intraglomerular pressure and activation of the renin-angiotensin aldosterone system (Forbes et al. 2007). Mechanisms underlying DN include altered renal blood flow autoregulation, with an early increase in the glomerular filtration rate due to afferent arteriolar vasodilation and efferent arteriolar vasoconstriction (Carmines 2010; Tuttle 2017). Diabetes-associated renal diseases are clinically detected by albuminuria (Ruggenenti et al. 2017). Renal angiotensin and dysregulated renal hemodynamics are attributed to diabetic kidney diseases, including fibrosis and inflammation (Tuttle 2017). In streptozotocin (STZ)-induced diabetes, the kidneys show DN changes, including glomerulosclerosis, tubulointerstitial fibrosis, associated with an increase in profibrotic proteins, collagen type IV, and transforming growth factor-beta 1 (TGF-β1) (Futrakul et al. 2007; Xu et al. 2008).

Valsartan is an angiotensin II (ANG II) receptor (AT1) blocker that antagonizes the vasoconstricting effect of AT1 on the glomerular efferent arterial vessels (Ruilope 2001) induced by diabetes (Carmines 2010; Tuttle 2017). Therefore, it reduces glomerular congestion and podocyte injury (Carmines 2010; Gagliardini et al. 2013; Magee et al. 2009; Patinha et al. 2013).
Aromatase catalyzes the conversion of testosterone into estradiol, and its expression is enhanced in diabetic kidney tissues (Prabhu et al. 2010; Xu et al. 2009). A mild increase in the low testosterone level in HG rats via enhancing endogenous testosterone by treatment with the aromatase inhibitor letrozole inhibited inflammatory cytokines and oxidative stress (Makary et al. 2017). In STZ-induced diabetic rats, inhibition of aromatase was associated with a decrease in estradiol (E2) and an increase in testosterone, and had a renoprotective against DN effect (Manigrasso et al. 2011).

In diabetic HG subjects, hemodynamic and non-hemodynamic processes, including inflammation and oxidative stress (Carmines 2010; Magee et al. 2009), are accompanied by low testosterone levels (Grossmann et al. 2008). This condition contributes to DN pathophysiology (Carmines 2010; Fukui et al. 2007; Magee et al. 2009). International guidelines state that testosterone levels should be routinely monitored in men with symptoms of primary HG that attend endocrine and diabetes clinics (Livingston et al. 2017).

The current study investigated the effect of blocking angiotensin signaling with valsartan in HG rats and tested whether correcting testosterone to the physiological level attenuates DN in this condition.
Material and Methods

Animals

In total, 80 adult male rats were used in this study. This research was carried out in accordance with the Guide to the Care and Use of Experimental Animals (2003), Canadian Council on Animal Care (CCAC), as previously described (Makary et al. 2017), and according to the institutional animal care guidelines. Animals were acclimatized for seven days. Throughout the experiments, rats had free access to water and food, and were housed at a controlled temperature, with 12-h light/dark cycles, as previously described (Makary et al. 2017).

Drugs and chemicals

Animals received a single intraperitoneal (i.p.) STZ injection (55 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). STZ was dissolved in 0.1 M citrate buffer (pH = 4.5) (Mankhey et al. 2005). Letrozole (0.5 mg/kg/day; Merck, Egypt) (Makary et al. 2017) and valsartan (30 mg/kg/day; Merck) (Sun et al. 2015; Zhang et al. 2015) were administered daily by oral gavage.

Animal models

The diabetic rat model was induced by subjecting the animals to overnight fasting and then giving them an i.p. injection of STZ. Only rats with confirmed diabetes as evidenced by blood sugar >300 mg/dL (day 3), measured from tail blood samples with a glucometer (OKmeter, OK Biotech, Taiwan), were included in the study. Diabetic animals received daily injections of insulin (subcutaneous, 2–4 U; Lantus, Sanofi-Aventis, Egypt) to maintain blood glucose levels at 250–400 mg/dL and to prevent weight loss (Kim et al. 2017). During the experiment (12 weeks), urine samples were collected to assess urine albumin excretion (UAE) and creatinine clearance (CrCl).
using metabolic cages. Blood pressure was measured using a BIOPAC non-invasive tail-cuff system (BIOPAC Systems, CA, USA) (Tawfik et al. 2018).

In diabetic rats, HG was induced by intramuscular injection of the luteinizing hormone-releasing hormone antagonist cetrorelix acetate (EMD Serono, Canada; 0.71 mg/kg/20 days) (Horvath et al. 2004). Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were assessed (Fig. 1B).

**Experimental design**

Rats were assigned to either one of two experiments, I and II, to test the HG model in diabetic rats and the renoprotective effect of letrozole and valsartan or their combination in STZ-induced diabetes, respectively.

*Experiment I* was designed to test the HG model with the diabetes effect on hormonal, inflammatory, and cytokines levels in this model. HG animals were compared to healthy and diabetic control animals (Fig. 1). Rats were allocated to four groups with 6 rats each: Group 1, CTL-normal-intact, non-diabetic (CTL-ND): normal rats injected with single i.p. STZ vehicle buffer solution. Group 2 intact, diabetic (CTL-D): diabetes induced by single STZ i.p injection. Group 3, HG- non-diabetic (HG-ND): rats received LHRH antagonist (cetrorelix) and non-diabetic. Group 4, HG- diabetic (HG-D): received STZ and cetrorelix.

*Experiment II* was designed to test the effects of letrozole, valsartan, and their combination in diabetic-HG animals on inflammatory cytokine levels and DN markers. HG animals that received letrozole and valsartan were compared to non-treated HG animals.

Rats were allocated to eight groups with 6-8 rats each: Group 1, CTL-HG- non-diabetic (CTL-HG-ND): rats injected with vehicle buffer; this group is considered the baseline for HG- non-

Letrozole and valsartan regimens were administered from day three, and rats were sacrificed after 12 weeks of treatment. Rats were euthanized, and blood samples were collected by cardiac puncture as described previously (Makary et al. 2017). Serum was collected by 10 min centrifugation. Serum samples and left kidneys were stored at −80 °C until use for various biochemical analyses. Right kidneys were weighed, fixed in 4% paraformaldehyde solution, and embedded in paraffin for histopathological assessment.

**Serum hormones, oxidative stress, and inflammatory markers assessments**

Serum concentrations of FSH, LH, testosterone (T), and estradiol (E2) were determined using commercial kits (Vidas, bioMérieux, France) (Makary et al. 2017). Left kidney samples were weighed and homogenized in buffer solution (pH = 7.8) using a Teflon homogenizer (Glas-Col; Vernon Hills, IL, USA). The homogenate was sonicated and centrifuged at 20,000 × g for 15 min. The supernatant was used for assessment of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GPx) levels spectrophotometrically using specific kits (Biodiagnostics, Egypt). Absorbance was measured using a UV-visible spectrophotometer (UV–1601PC; Shimadzu, Japan). The kidney samples were further processed for assaying IL-1β, IL-6, and TNF-α concentrations using enzyme-linked immunosorbent assay (ELISA) kits (R&D
Renal function assessment
Renal dysfunction was assessed by measuring serum levels of blood urea nitrogen and creatinine using a Bioclin kit (Santa Coloma, Spain). Urine samples were collected for 24 h using metabolic cages. CrCl was calculated as follows: 
\[ \text{CrCl} = \frac{\text{urine creatinine} \times \text{urine volume}}{1440 \times (\text{serum creatinine} \times \text{time})} \] (Shokeir et al. 2012). UAE was measured by using microalbumin kits strip (Nephelometry BN ProSpec, Siemens HealthCare Diagnostic Products, Germany) (Ndisang and Jadhav 2014). The urine cystatin C level was measured using ELISA (R&D Systems, MN, USA) (Togashi and Miyamoto 2013), according to the manufacturer’s guidelines.

Blood glucose assessment
Every three days and on the day before sacrifice, blood samples collected from the tail vein after an overnight (16-h) fast and tested by the OKmeter glucometer. Animals with fasting blood glucose >300 mg/dL were considered diabetic (Masoad et al. 2012). Glycemic control was assessed by measuring HbA1c by high-performance liquid chromatography (Ruggenenti et al. 2017). Random blood sugar was assessed using the OKmeter.

Quantitative reverse-transcription (RT-q)PCR
RNA was extracted from homogenized renal tissue using a Promega RNA isolation kit (Promega, Madison, WI, USA). The RNA concentration was determined using an ultraviolet spectrophotometer. cDNA was prepared from 1 μg RNA using the SuperScript III First-Strand
Synthesis kit (#K1621; Fermentas, Waltham, MA, USA). Gene Runner Software (Hasting Software, Hasting, NY, USA) was used to design primers targeting NF-κB on the basis of GenBank RNA sequences. The gene-specific primer pair for NF-κB was: forward, 5'-CATTGAGGTTATTTATCCG-3', reverse, 5'-GGCAAGTGGCCATTGTGTC-3'. cDNA was amplified by qPCR using SYBR Green Master Mix and data were analyzed using Applied Biosystems software version 3.1 (StepOne™, USA). Relative target gene expression was calculated by the comparative (Ct) method. Results were normalized to the level of β-actin and reported as a fold change.

Renal angiotensin II, collagen IV, and TGF-β

ANG II was evaluated by ESILA using rat ANG II (BioVendor, Brno, Czech Republic). Collagen IV and TGF-β1 contents were measured in renal homogenates using rat collagen IV and TGF-β1 kits (CUSABIO Technology LLC, TX, USA) according to the manufacturer’s guidelines.

Renal diabetic nephropathy histopathological evaluation

Renal damage was evaluated and scored according to the grading system reported by Tervaert et al. (Tervaert et al. 2010), with slight modification. Glomerular expansion, renal tubules and interstitial desquamation, cytoplasmic changes as hydropic degeneration, and peritubular and interstitial inflammatory infiltration were evaluated using hematoxylin and eosin-stained (H&E) slides. Periodic acid-Schiff (PAS) stain was used to evaluate the presence of glomerular and tubular hyaline membrane thickening. Masson’s trichome stain was used to semi-quantitatively evaluate interstitial fibrosis and vascular wall thickening. Scoring was as follows: in grade 0, there is abnormality detected in glomeruli or tubules. In grade 1, there is glomerular damage that is scored
according to thickening in glomerular thickness, mesangial hypercellularity, with no interstitial or tubular inflammation. In grade 2, there is presence of significant enlargement in the glomerulus, with obliteration of capillary lumens, loss of lobulation, and prominent thickening in the glomerular wall. The mesangium shows expansion with diffuse sclerosis. These changes involve less than 50% of glomeruli, with no interstitial or tubular inflammation. Grade 3 is similar to grade 2, but changes involve more than 50% of the glomeruli, with interstitial and tubular inflammation.

**Statistical analysis**

Data were expressed as the mean ± SD and were analyzed using SPSS version 17 (IBM, Armonk, NY, USA). Means were compared by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple comparisons. \( P < 0.05 \) was considered significant.

**Results**

Experiment I

**Serum testosterone and estradiol levels in normal and HG adult male rats in response to diabetes**

Experiment I: adult male rats were administered a low dose of the luteinizing hormone-releasing hormone antagonist cetrorelix pamoate to induce the HG state. Cetrorelix decreased serum testosterone and E2 levels at 12 weeks in diabetic (HG-D) as well as non-diabetic (HG-ND) adult male rats when compared to their respective controls \( \left( P < 0.05; \text{Fig. 1} \right) \). Diabetes also decreased serum testosterone, by 42.94% and 57.81% in CTL-D and HG-D groups, respectively \( \left( \text{Fig. 1A; } P < 0.05 \right) \). Serum E2 by was increased by 31.86% and 68.03% in CTL-D and HG-D groups, respectively, when compared to their respective controls \( \left( \text{Fig. 1B; } P < 0.05 \right) \). Serum FSH and LH
showed no significant changes (Fig. 1C, D). These results show the diabetes-induced hypoandrogenic effect that augmented with HG.

**Diabetes enhances renal inflammatory cytokine and oxidative stress levels in HG rats**

Diabetes affects renal oxidative stress and inflammatory cytokines (Anderberg et al. 2015; Brahler et al. 2012). To determine if HG and diabetes (experiment I) affect oxidative stress and inflammatory cytokines, their levels were measured in renal homogenate. Diabetes enhanced oxidative stress burden as indicated by the significantly higher MDA and lower SOD and GPx levels in CTL-D than in CTL-ND animals ($P < 0.05$; Fig. 2A–C). HG-D animals were affected more than CTL-D group ($P < 0.05$; Fig 2 A-C). Diabetes enhanced oxidative stress which intern activates inflammatory cytokines (Cao and Cooper 2011). Based on this, inflammatory cytokines were tested in the study groups. The HG-D group showed enhanced inflammatory cytokines IL-1β, IL6, and TNFα when compared to the CTL-D group ($P < 0.05$; Fig. 3) confirming diabetes-augmented renal oxidative stress and inflammatory conditions.

**Experiment II**

**Effects of letrozole and valsartan on serum testosterone, E2, FSH, LH levels in HG animals**

Previous results of experiment I showed an increase in renal inflammatory and oxidative stress in HG-D rats group. In experiment II, letrozole as an enhancer of endogenous testosterone and anti-inflammatory properties was tested. Diabetes also induces renal efferent arteriole vasoconstriction. Therefore, valsartan renal vasodilatory effect was tested in CTL HG-D rats.
The effects of letrozole and valsartan on serum testosterone, E2, FSH, and LH levels were studied in HG-ND and HG-D rats (Table 1). At the end of the experiment (week 12), diabetes decreased serum testosterone in CTL HG-D compared to CTL HG-ND rats ($P < 0.05$; Table 1). Meanwhile, diabetes increased E2 levels in the same groups ($P < 0.05$; Table 1). Letrozole and letrozole+valsartan HG-treated groups increased serum testosterone compared to CTL HG-D animals ($P < 0.05$, Table 1). Meanwhile, letrozole and letrozole+valsartan reduced serum E2 in the HG-treated groups compared to CTL HG-D animals ($P < 0.05$, Table 1). Valsartan alone showed no effect on these parameters. Both letrozole and valsartan induced similar responses in CTL-ND groups, but did not show any effect on serum FSH or LH. The current results showed the effect of the letrozole in ameliorating the low testosterone level in CTL HG-D rats.

**Effects of letrozole and valsartan on renal physiological parameters**

Because the observed increase in serum testosterone by letrozole in HG rats (experiment II, Table 1), which decreased significantly in HG rats (Fig. 1), we evaluated both letrozole and valsartan effects on renal function parameters (Table 2) in HG-ND and HG-D rats groups. The mean values of renal function parameters, including serum blood urea nitrogen, creatinine, and cystatin C in CTL HG-D rats were significantly increased compared to the CTL HG-ND group ($P < 0.05$, Table 2). Meanwhile, there was a decrease in calculated CrCl in CTL HG-D compared to CTL HG-ND animals ($P < 0.05$, Table 2). Letrozole and letrozole + valsartan HG-treated groups significantly improved renal function when compared the levels in the CTL HG-D group ($P < 0.05$, Table 2). The combined treatment improved testosterone level and ameliorated renal vasoconstriction which was reflected on renal function parameters.
UAE response to letrozole and valsartan in HG rats

Based on experiment II, letrozole and valsartan showed an improvement in renal function (Table 2). As is shown in Table 3, after 12 weeks of diabetes, CTL HG-D animals had a significant increase in UAE (from 3.1 ± 0.37 mg/day to 123.1 ± 23.76 mg/day, \( P < 0.05 \); Table 3). Letrozole- and valsartan HG-treated groups showed significant reductions in UAE levels at 12 weeks (32.33 ± 6.50 mg/day and 25.95 ± 4.59 mg/day, \( P < 0.05 \); Table 3) compared to at 0 weeks. The HG-D/LET+VAL group had a significantly lower UAE level at 12 weeks (14.60 ± 3.68 mg/day, \( P < 0.05 \); Table 3) than at 0 weeks, and the level was significantly lower than that at 12 weeks in the HG-D/letrozole group (\( P < 0.05 \)). Thus, in CTL HG-D animals improving the low testosterone level and decreasing the arteriolar vasoconstriction improved the UAE excretion as a marker of DN.

Effects of letrozole and valsartan on body and kidney weights, blood pressure, and glycemia

In CTL HG-D rats improvement of renal functions (Table 2) and UAE (Table 3) by letrozole and valsartan were obvious. Therefore, the effects of letrozole and valsartan treatment on general and systemic parameters as body, kidney weights, blood pressure and glycemia in HG-ND and HG-D rats at 12 weeks are shown in Table 4. CTL HG-D animals had a significant increase in the kidney-to-body weight ratio (0.37% at 0 weeks vs. 1.62% at 12 weeks, \( P < 0.05 \); Table 4). The HG-D/LET, and HG-D/VAL, and HG-D/LET+VAL groups showed significant reductions in the kidney-to-body weight ratio (\( P < 0.05 \); Table 4) compared to the CTL HG-D group. Letrozole, valsartan, and letrozole + valsartan decreased systolic blood pressure by 10.79%, 25.46%, and 28.48%, respectively, when compared to the pressure in CTL HG-D rats (\( P < 0.05 \), Table 4). A similar effect was observed in diastolic blood pressure. No effect of letrozole or valsartan on the glycemic level was detected. Although both drugs had no effect on the glycemic level, they were able to improve
the systemic parameters. This comes in agreement with the observed improvement in renal functions and UAE.

**Effects of letrozole and valsartan on renal NF-κB correlation with HbA1c and ANG II**

The observed hyperglycemia (Table 4) was confirmed by the increase in HbA1c level (Table 5), as long term hyperglycemia marker, with no significant difference between the studied groups. However, letrozole, valsartan, letrozole + valsartan treatment resulted in significant reductions in the NF-κB level when compared to the level in the CTL HG-D group ($P < 0.05$, Table 5). Letrozole + valsartan had a significantly stronger effect than either letrozole or valsartan treatment ($2.59 \pm 0.87$ vs. $9.08 \pm 2.84$ and $5.24 \pm 1.89$, respectively, $P < 0.05$; Table 5). The effects of the drug treatments correlated with the reduction in NF-κB expression in CTL HG-D rats ($R^2 = 0.92, 0.96$ and $0.88$, respectively, $P < 0.05$; Fig. 4). Letrozole and valsartan without affecting hyperglycemia were able to reduce the renal NF-κB level in HG animals.

Valsartan is ANG II blocker. Based on the reducing effect of the letrozole and valsartan on renal NF-κB level, the effect of the drugs on renal ANG II was evaluated in HG-ND and HG-D animals. The HG-D/VAL and HG-D/LET+VAL groups, but not the HG-D/LET group, showed a significant decrease in ANG II when compared to the CTL HG-D group (HG-D/VAL, $63.63 \pm 22.17$, HG-D/LET +VAL, $38.63 \pm 9.49$, HG-D/LET, $149.9 \pm 26.57$, HG-D, $161.9 \pm 13.05$, $P < 0.05$; Fig. 5). The combination of letrozole + valsartan treated HG-D rats more strongly reduced renal ANG II content than HG-D/VAL-treated rats ($P < 0.05$; Fig. 5). The drug combination treated groups made no significant difference between HG-ND and HG-D ($P > 0.05$; Fig. 5). The result shows the valsartan ameliorating effect of diabetes-induced renal ANG II in HG-D animals.
Letrozole and valsartan attenuate oxidative stress in HG diabetic rats

Experiment I showed an increase in the oxidative stress in HG-D rats (Fig. 2). Here, the effect of letrozole and valsartan with their anti-inflammatory and vasodilatory effects, respectively, were assessed (Experiment II, Fig. 6). Diabetes induced oxidative stress in CTL HG-D renal tissues, as indicated by the significant increases in MDA levels associated with decreases in SOD and GPx levels in CTL HG-D compared to in CTL HG-ND rats ($P < 0.05$; Fig. 6A–C). The oxidative stress effect was significantly reduced by letrozole, valsartan, or their combination in HG-D-treated rats ($P < 0.05$; Fig. 6A–C). Letrozole + valsartan-treated rats ameliorated oxidative stress difference between HG-D and HG-ND groups ($P > 0.05$; Fig. 6).

**Letrozole and valsartan reduce inflammatory cytokines in hypogonadal diabetic rats**

Experiment I showed an increase in the inflammatory cytokines in HG-D rats (Fig. 3). Letrozole and valsartan reduced the renal inflammatory marker NF-κB (Experiment II, Fig. 4 and Table 5). Here, the ameliorating drug effects were tested in HG rats. The inflammatory cytokines IL-1β, IL6 and TNFα (Fig. 7A–C) were significantly enhanced by diabetes in the CTL HG-D compared to the CTL HG-ND groups ($P < 0.05$). Letrozole alone or in combination with valsartan significantly reduced the diabetic-induced increase in cytokines ($P < 0.5$; Fig. 7). In HG-D treated groups, the combination treatment had a stronger ameliorating effect than letrozole or valsartan treatment alone ($P < 0.05$). Letrozole + valsartan ameliorated the difference of renal IL-1β and TNFα between the HG-D compared to the HG-ND-treated groups ($P > 0.05$; Fig. 7) denoting that diabetes-induced low testosterone and renal arteriolar vasoconstriction contributed to the increase in the oxidative stress and consequently the inflammatory cytokines in HG rats.
Letrozole and valsartan improve HG-diabetic-induced histopathological changes in the kidneys

Histopathological examination of renal tissues revealed that in CTL HG-ND rats, renal glomeruli and tubules architecture were preserved (H&E; Fig. 8 A-I). However, in the CTL HG-D group, renal tissues exhibited significant enlargement in the glomerulus, with obliteration of capillary lumens, loss of lobulation and prominent thickening in the glomerular wall (Fig. 8 A,B-II). The mesangium was expanded, with diffuse sclerosis, tubules obliteration (PAS; Fig. 8 A,B-II) and glomerular and extracellular matrix accumulation (Masson; Fig. 8 B,C-II). These changes involved more than 50% of the glomeruli (Fig. 8; H&E, PAS, Masson’s trichrome). Letrozole, valsartan, and their combination significantly reduced HG-D-induced glomerular changes and histopathological scores (CTL HG-D, 2.25 ± 0.89; HG-D/LET, 0.75 ± 0.71; HG-D/VAL, 1.00 ± 1.01; HG-D/LET+VAL, 0.63 ± 0.74; \( P < 0.5 \); Fig. 9A). This result indicates the improvement in inflammation and vasodilation by letrozole and valsartan improves the DN changes in HG rats.

Effects of letrozole and valsartan on collagen IV and TGF-β1 contents in HG-diabetic rats

Histopathology revealed increase in DN changes in HG-D rats (Fig. 8A-C). Therefore, we assessed two of the DN elements, collagen IV and TGF-β1, contributing to the fibrotic changes. Collagen IV and TGF-β1 contents were increased in CTL HG-D (Figs. 9A and 10A). Letrozole, valsartan, and their combination reduced the diabetic-induced increases in collagen IV and TGF-β1 (\( P < 0.5 \); Figs. 9 and 10). The combined treatment had a stronger effect on collagen IV reduction than letrozole or valsartan treatment alone in HG-D-treated rats compared to CTL HG-D group (LET, 45.5%, VAL, 55.56%, LET+VAL, 75.25%, respectively; \( P < 0.05 \)). Similar patterns were observed for TGF-β1 contents after letrozole or valsartan versus the combined treatment (\( P < 0.05 \) in HG-
D-treated compared to CTL-HG-D rats (Fig. 10). Thus, correcting low testosterone in HG-D animals, by letrozole, and ameliorating diabetes-induced arteriolar vasoconstriction, by valsartan, improved collagen IV and TGF-β1 and their contribution to the glomerular fibrosis and DN.

**Discussion**

The findings of the current study can be summarized as follows: in HG diabetic rats, valsartan alone or in combination with letrozole (a) improved renal functions and morphology, (b) suppressed ANG II activation, (c) improved oxidative stress and inflammatory cytokines, and (d) improved collagen IV and TGF-β1 levels, which correlated with renal histopathological changes, without affecting hyperglycemia. Although the mode of action of valsartan, ANG II blockade, differs from that of letrozole, testosterone enhancer, both induce a reduction in the renal diabetic insult via different mechanisms. High blood glucose induces ANGII and NFκB, which activate inflammatory cytokines and thus favor glomerular fibrosis (Suryavanshi and Kulkarni 2017). Valsartan, through its vasorelaxant effect on the efferent arteriole, prevents glomerular hypertension, which initiates DN (Carmines 2010; Ruilope 2001; Tuttle 2017), through a reduction in TGFβ1. TGFβ1 is implicated in the harmful effects of ANG II on mononuclear cell infiltration, proinflammatory cytokines, oxidative stress, and consequently, tubulointerstitial injuries and sclerotic glomeruli (Ruilope 2001; Wiecek et al. 2003). Letrozole, through its role in enhancing endogenous testosterone via its immunomodulatory effect, attenuates the diabetes-associated inflammatory condition and consequently, DN (Anderberg et al. 2015; Brahler et al. 2012. Maggio, 2005 #34; Malkin et al. 2004; Manigrasso et al. 2012; Vodo et al. 2013).

HG is a clinical syndrome that involves abnormally low physiological levels of testosterone due to disruption of the hypothalamic-pituitary-gonadal axis at one or more levels (Cunningham
and Toma 2011). Late-onset HG is common among men over the age of 40 years (Livingston et al. 2017) and in patients with end-stage renal disease, which is associated with diabetes (Bao and Johansen 2015). Further, diabetes and higher body mass index are associated with HG (Anderson et al. 2012; Khurana et al. 2014).

DN involves renal reaction to hyperglycemia and associated hemodynamic changes that contribute to the production of ROS and the proinflammatory cytokines IL-1β, IL-6 and TNFα (Anderberg et al. 2015; Brahler et al. 2012; Erejuwa et al. 2011; Manigrasso et al. 2012; Maritim et al. 2003; Ndisang and Jadhav 2014; Patil et al. 2016), which was corroborated by our findings. When serum testosterone decreases due to diabetes per se (Xu et al. 2009; Xu et al. 2008) or on top of HG, it accelerates deterioration of the renal functions and enhances glomerulosclerosis. The current study showed that, in HG with a partial decrease in testosterone, diabetes resulted in deterioration of the renal structure and function, similar to findings in castrated animals with zero-level testosterone (Prabhu et al. 2010; Sun et al. 2007; Xu et al. 2009; Xu et al. 2008). These findings indicate the importance of a normal testosterone level in men (Manigrasso et al. 2012).

Aromatase converts testosterone to E2 in gonadal and extragonadal tissues, and its inhibition by letrozole increases the testosterone level (Manigrasso et al. 2012; Manigrasso et al. 2011; Prabhu et al. 2010; Xu et al. 2009). Letrozole, but not valsartan, enhances testosterone and suppressed E2 levels, without affecting FSH and LH levels (Makary et al. 2017), as was also observed in the current study. Inhibition of aromatase in addition to dihydrotestosterone treatment ameliorated DN in STZ diabetic rats (Manigrasso et al. 2012). In intact and castrated STZ diabetic rats, low doses of dihydrotestosterone successfully decreased DN parameters (Xu et al. 2009). In castrated rats, the testosterone is almost zero (Sun et al. 2007), unlike in HG, where there is a partial decrease in testosterone (Horvath et al. 2004). In the current study, in HG rats, the aromatase
inhibitor letrozole attenuated DN parameters, similar to findings by Manigrasso et al. (Manigrasso et al. 2011) in intact rats. Thus, enhancing endogenous testosterone to normal physiological levels has a renoprotective effect against DN in male HG rats.

Androgen-regulated protein is localized to the proximal convoluted tubules and is secreted into extracellular tissue, which plays a role in the protection against the inflammatory condition associated with diabetes (de Quixano et al. 2017). The mild testosterone induction in the current study was not comparable to the effect of supraphysiological testosterone replacement (Dousdampanis et al. 2014). Testosterone has profibrotic properties (Dousdampanis et al. 2014), worsening glomerulosclerosis. Testosterone also has a vasorelaxant effect (Marrachelli et al. 2010; Ramirez-Rosas et al. 2011). Diabetes-associated hemodynamic and non-hemodynamic changes are due to increased renal glycemia, which affects the renal vascular bed, causing glomerular hyperfiltration and disrupting the glomerular filtration barrier via local ANG II release (Carmines 2010; Magee et al. 2009; Patinha et al. 2013; Satchell and Tooke 2008; Tuttle 2017; Vidotti et al. 2004). The latter can be detected by an increase in albuminuria, ANG II, or cystatin C (Peterson et al. 2017; Ruggenenti et al. 2017; Togashi and Miyamoto 2013). A dysfunctional barrier induces an increase in UAE, a hallmark of DN (Manigrasso et al. 2011; Xu et al. 2009; Xu et al. 2008), and cystatin C, as shown for HG diabetic rats in the current study. The immunomodulatory effect of letrozole via testosterone could improve creatinine and CrCl, as observed previously for the macrophage inhibitor, hemin (Ndisang and Jadhav 2014). However, valsartan (Currie et al. 2017), through its hemodynamic effects, had a stronger renal protective effect on HG DN when used alone or in combination with letrozole.

We detected an increase in ANG II in diabetic rats, which can induce hemodynamic changes (hyperfiltration) and inflammatory processes leading to the activation of IL-1, TNFα, and
profibrogenic TGF-β1, as well as collagen IV, which is associated with DN (Russo et al. 2007; Sanajou et al. 2018; Wiecek et al. 2003). The current study revealed a correlation between HbA1c and NF-κB expression and increase in ANG II in HG-diabetic animals, which indicates the importance of hyperglycemia and local ANG II release in inducing DN and enhancing NF-κB expression. ANG II and NF-κB initiate a, inflammatory cascade, including IL-1, TNFα, TGF-β, and collagen expression, together with oxidative stress (Wiecek et al. 2003). The current results showed that blocking the action of ANG II by valsartan alone or in combination with letrozole attenuated NF-κB expression in HG diabetic rats. Consequently, valsartan and letrozole halted profibrotic cytokine expression and fibrosis in these rats. Furhter, an ANG II blocker can reduce the increase in blood pressure observed in DN (Bunag et al. 1982; Cadaval et al. 2000; Rossing et al. 2003), as observed in the HG-diabetic rats in this study.

Conclusion

The present study demonstrated that in HG rats, diabetes-induced DN and the decline in renal function are caused by multiple defects. HG enhances renal vulnerability to hyperglycemia by enhancing renal NFκB and ANG II, consequently leading to profibrotic changes. Restoring the physiological testosterone level and blocking of ANG II by letrozole and valsartan, respectively, attenuated DN processes in HG male STZ-induced diabetic rats. These data highlight the importance of physiological levels of testosterone and ANG II in the pathophysiology of DN in the HG male state. Therefore, screening serum testosterone in diabetic and/or aged men is warranted. The findings of our study are valuable to diabetic subjects with HG, for whom regulation of the renin-angiotensin system via angiotensin receptor blockers might improve the prognosis of chronic diabetic-related kidney disease.

Conflict of interest
The authors declare that there are no conflicts of interest associated with this study.

Acknowledgment

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Tawfik, M.K., El-Kherbetawy, M.K., and Makary, S. 2018. Cardioprotective and Anti-Aggregatory Effects of Levosimendan on Isoproterenol-Induced Myocardial Injury in High-Fat-Fed Rats Involves Modulation of PI3K/Akt/mTOR Signaling Pathway and Inhibition of Apoptosis:


https://mc06.manuscriptcentral.com/cjpp-pubs


Table 1: Effect of letrozole and valsartan on hormonal parameters in hypogonadal diabetic and non-diabetic adult male rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>T (ng/mL)</th>
<th>E2 (pg/mL)</th>
<th>FSH (mIU/mL)</th>
<th>LH (mIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL HG-ND N=6</td>
<td>1.45±0.22</td>
<td>4.98±1.30</td>
<td>4.23±0.65</td>
<td>3.08±1.42</td>
</tr>
<tr>
<td>HG-ND/ LET N=6</td>
<td>3.10±0.59*</td>
<td>1.65±0.52*</td>
<td>4.80±1.14</td>
<td>3.40±1.44</td>
</tr>
<tr>
<td>HG-ND/ VAL N=6</td>
<td>1.53±0.18*</td>
<td>4.05±1.55*</td>
<td>3.87±1.40</td>
<td>2.42±1.06</td>
</tr>
<tr>
<td>HG-ND/ LET+ VAL N=6</td>
<td>3.30±0.56*</td>
<td>1.62±0.56*</td>
<td>4.38±1.48</td>
<td>3.03±1.16</td>
</tr>
<tr>
<td>CTL HG-D N=8</td>
<td>0.74±0.25*</td>
<td>6.94±1.45*</td>
<td>3.93±1.72</td>
<td>2.84±1.29</td>
</tr>
<tr>
<td>HG-D/ LET N=8</td>
<td>2.37±0.44**</td>
<td>3.31±0.48*</td>
<td>4.24±2.15</td>
<td>3.46±1.69</td>
</tr>
<tr>
<td>HG-D/ VAL N=8</td>
<td>1.21±0.31</td>
<td>7.06±1.36*</td>
<td>5.03±1.68</td>
<td>3.28±1.59</td>
</tr>
<tr>
<td>HG-D/ LET+ VAL N=8</td>
<td>2.29±0.54*</td>
<td>3.96±0.89*</td>
<td>4.61±1.61</td>
<td>2.98±1.55</td>
</tr>
</tbody>
</table>

Experiment II, note: Values are the mean ± SD of hypogonadal (HG) non-diabetic (ND) and diabetic (D) rats in control (CTL) condition and with treatment. One-way ANOVA and post hoc Tukey’s tests were done between different groups regarding serum T, testosterone; E2, estradiol; FSH, follicular stimulating hormone; LH, luteinizing hormone of the studied groups on week 12 before sacrifice. Values were estimated in the HG adult male rats induced by cetrorelix, 0.71 mg/Kg every 20 day with and without letrozole, LET and valsartan, VAL treatment. For HG-D
corresponding values, T and E2 level comparisons; \#P < 0.05 vs. CTL HG-D rats; †P < 0.05 vs.
HG-D/ LET rats; $P < 0.05 vs. HG-D/ VAL rats; ‡P<0.05 HG-D/ LET + VAL. There was no
significant difference between T or E2 levels of HG-D/ LET and HG-D/ LET+VAL, P>0.05.
For HG-ND, T and E2 level comparisons; *P < 0.05 vs. CTL HG-ND rats; ‖P< 0.05 vs. HG-
ND/VAL; ¶P<0.05 vs HG-ND/ LET + VAL. No significant difference between FSH and LH
between groups. N, number of rats.
Table 2: Effect of letrozole and valsartan on renal physiological parameters in hypogonadal diabetic and non-diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood urea nitrogen (mg/dL)</th>
<th>Serum Creatinine (mg/dL)</th>
<th>Creatinine Clearance (mL/min)</th>
<th>Urine Cystatin C (μg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL HG-ND N=6</td>
<td>27.33± 2.07</td>
<td>0.82±0.08</td>
<td>4.63±0.68</td>
<td>2.63±0.67</td>
</tr>
<tr>
<td>HG-ND/ LET N=6</td>
<td>26.67± 3.88†</td>
<td>0.86±0.13‰</td>
<td>4.34±0.85†</td>
<td>2.55±0.48†</td>
</tr>
<tr>
<td>HG-ND/ VAL N=6</td>
<td>27.50± 3.45†</td>
<td>0.78±0.10‰</td>
<td>4.30±1.07†</td>
<td>2.38±0.66†</td>
</tr>
<tr>
<td>HG-ND/ LET+ VAL N=6</td>
<td>28.00± 5.48†</td>
<td>0.80±0.11‰</td>
<td>4.16±0.86†</td>
<td>2.54±0.80†</td>
</tr>
<tr>
<td>CTL HG-D N=8</td>
<td>92.25± 25.88*</td>
<td>5.90±0.68*</td>
<td>1.38±0.27*</td>
<td>13.74±5.21*</td>
</tr>
<tr>
<td>HG-D/ LET N=8</td>
<td>70.25± 11.20*&amp;&amp;†</td>
<td>3.15±0.61*&amp;&amp;</td>
<td>2.80±0.56*&amp;&amp;</td>
<td>6.75±1.39*&amp;&amp;</td>
</tr>
<tr>
<td>HG-D/ VAL N=8</td>
<td>47.50±15.76&amp;&amp;</td>
<td>2.26±0.53*&amp;&amp;</td>
<td>3.32±0.37*&amp;&amp;</td>
<td>3.76±0.56*&amp;&amp;</td>
</tr>
<tr>
<td>HG-D/ LET+ VAL N=8</td>
<td>29.50±6.53&amp;&amp;†</td>
<td>1.13±0.44*&amp;&amp;†</td>
<td>4.11±0.42*&amp;&amp;†</td>
<td>2.70±0.92*&amp;&amp;</td>
</tr>
</tbody>
</table>

Experiment II, note: Values are the mean ± SD of hypogonadal (HG) non-diabetic (ND) and diabetic (D) rats in control (CTL) condition and with reatment. One-way ANOVA and post hoc Tukey’s tests were done between different renal parameters of studied groups on sacrifice day after 12 weeks. Letrozole, LET and valsartan, VAL treated HG rats model was induced by cetorelix, 0.71 mg/Kg every 20 day. *P<0.05 for corresponding parameters: BUN, Cr, CrCl and urine cystatin C groups in HG-D rats; **P < 0.05 vs. HG-ND rats; †P < 0.05 vs. CTL HG-D rats;
†$P < 0.05$ vs. HG-D/LET rats; $\$P < 0.05$ vs. HG-D/VAL rats. For all the studied renal parameters there was non-significant association between ($P > 0.05$) HG-D/LET + VAL vs. HG-D/VAL rats. N, number of rats.
Table 3: Effect of letrozole and valsartan on urea/albumin excretion over 12 weeks in hypogonadal adult male rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 week</th>
<th>4 week</th>
<th>8 week</th>
<th>12 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL HG-ND (n=6)</td>
<td>2.85±0.47</td>
<td>3.23±0.68</td>
<td>4.2±1.42</td>
<td>5.07±1.73</td>
</tr>
<tr>
<td>CTL HG-D (n=8)</td>
<td>3.1±0.73</td>
<td>19.88±7.36</td>
<td>58.13±27.59*</td>
<td>123.1±23.76**</td>
</tr>
<tr>
<td>HG-D/LET (n=8)</td>
<td>3.19±0.71</td>
<td>14.25±4.03</td>
<td>24.63±10.725**#</td>
<td>32.33±6.501**‡</td>
</tr>
<tr>
<td>HG-D/VAL (n=8)</td>
<td>4.38±3.11</td>
<td>13.5±4.72</td>
<td>19.38±11.46#</td>
<td>25.95±4.591*‡</td>
</tr>
<tr>
<td>HG-D/LET + VAL (n=8)</td>
<td>4.49±3.12</td>
<td>8.00±2.20</td>
<td>11.63±2.39#</td>
<td>14.6±3.68#</td>
</tr>
</tbody>
</table>

Experiment II, groups: CTL, control; HG, hypogonadal; ND, non-diabetic; D, Diabetic which were received no treatment or LET, letrozole and VAL, valsartan. One-way ANOVA and post hoc Tukey’s tests were done between (i) between different weeks for the same group (ii) between different groups of the same week,

(i) For the same group; $P < 0.05$ at 8 week; ‖$P<0.05$ at 12 week compared to 0 week.

(ii) For the same week, *$P < 0.05$ vs. control CTL HG-ND rats; #$P < 0.05$ vs. CTL HG-D rats; ‡$P < 0.05$ HG-D/LET vs. HG-D/LET + VAL rats. $P > 0.05$ (non-significant) HG-D/VAL vs HG-D/LET +VAL rats. N, number of rats.
Table 4: Characteristics of hypogonadal diabetic and non-diabetic adult male rats in response to letrozole and valsartan.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Body weight (g)</th>
<th>Wet kidney weight (g)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Diastolic blood pressure (mmHg)</th>
<th>Glycemia (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL HG-ND N=6</td>
<td></td>
<td>292.5± 12.47</td>
<td>1.08±0.16</td>
<td>121.3±2.81</td>
<td>75.83±4.75</td>
<td>83.33±5.65</td>
</tr>
<tr>
<td>HG-ND/ LET N=6</td>
<td></td>
<td>307.30±27.82</td>
<td>1.11±0.15</td>
<td>120.5±5.51</td>
<td>71.83±3.49</td>
<td>73.67±8.80†‡</td>
</tr>
<tr>
<td>HG-ND/ VAL N=6</td>
<td></td>
<td>296.70± 32.09</td>
<td>1.01±0.13</td>
<td>115.7±2.88</td>
<td>64.17±2.79</td>
<td>77.00±9.78†‡</td>
</tr>
<tr>
<td>HG-ND/ LET+ VAL N=6</td>
<td></td>
<td>320.3±42.57†‡</td>
<td>1.04±0.19</td>
<td>117.0±3.35†‡</td>
<td>67.50±2.43†‡</td>
<td>73.33±9.31†‡</td>
</tr>
<tr>
<td>CTL HG-D N=8</td>
<td></td>
<td>255.0± 16.48*</td>
<td>4.31±0.85</td>
<td>172.4±13.88*</td>
<td>88.38±3.38*</td>
<td>339.0±39.45*</td>
</tr>
<tr>
<td>HG-D/ LET N=8</td>
<td></td>
<td>262.1± 40.27</td>
<td>1.07±0.15</td>
<td>153.8±12.83</td>
<td>79.88±4.16‡</td>
<td>333.1±55.15*</td>
</tr>
<tr>
<td>HG-D/ VAL N=8</td>
<td></td>
<td>253.6± 35.17</td>
<td>0.94±0.21</td>
<td>128.5±8.02‡</td>
<td>73.63±2.72‡</td>
<td>342.3±53.80*</td>
</tr>
<tr>
<td>HG-D/ LET+ VAL N=8</td>
<td></td>
<td>265.5±39.67</td>
<td>1.23±0.09</td>
<td>123.3±5.23‡</td>
<td>69.88±7.99‡</td>
<td>333.3±38.50*</td>
</tr>
</tbody>
</table>

Experiment II, note: Values are the mean ± SD of hypogonadal (HG) non-diabetic (ND) and diabetic (D) rats in control (CTL) and treatment conditions. One-way ANOVA and post hoc Tukey’s tests were done between different groups regarding body and kidney weights, systolic and diastolic blood pressure, and hyperglycemia on week 12 before sacrifice. Values were estimated in HG adult male rats induced by cetrorelix, 0.71 mg/Kg every 20 day with and without letrozole,
LET and valsartan, VAL treatment. For corresponding values; *$P < 0.05$ vs. CTL HG-ND rats; #$P < 0.05$ vs. CTL HG-D rats; †$P < 0.05$ vs. HG-D/ LET rats; $P < 0.05$ vs. HG-D/ VAL rats; ‡$P < 0.05$ vs. HG-D/ LET + VAL.
Table 5: Effect of letrozole and valsartan on NFκB and glycated hemoglobin (A1c) in hypogonadal (HG) diabetic adult male rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>NFκB</th>
<th>(Hb)A1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL HG-D (n=8)</td>
<td>16.58±3.05</td>
<td>11.17±1.17</td>
</tr>
<tr>
<td>HG-D/ LET (n=8)</td>
<td>9.08±2.84*</td>
<td>10.56±2.12</td>
</tr>
<tr>
<td>HG-D/ VAL (n=8)</td>
<td>5.24±1.89##</td>
<td>11.25±1.60</td>
</tr>
<tr>
<td>HG-D/ LET+ VAL (n=8)</td>
<td>2.59±0.87##</td>
<td>10.44±2.40</td>
</tr>
</tbody>
</table>

Experiment II, note: Values are the mean ± SD. CTL, control; D, diabetes; HG, hypogonadism. N, number of rats. One-way ANOVA and post hoc Tukey’s tests were done between groups on sacrifice day. For NFκB and glycated hemoglobin, A1c; *P < 0.05 vs. CTL HG-D; #P < 0.05 vs. HG-D/ LET. P > 0.05 (non-significant) HG-D/VAL vs HG-D/LET +VAL.
**Figure caption**

**Fig. 1 Effect of hypogonadism on serum hormonal concentrations in diabetic and non-diabetic adult male rats.**

Experiment I: Hypogonadism effect in non-diabetic (ND) and diabetic (D) rats is showed. A, Diabetes decreased serum testosterone and B, increased estradiol (E2) levels in control intact (CTL) and hypogonadal rats (HG) groups. C and D, there was no difference of serum follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels between groups. Hypogonadism was induced by LHRH, luteinizing hormone-releasing hormone antagonist (cetrorelix, 0.71 mg/Kg every 20 days). Number of animals (N) per group is six. Values are the mean ± SD. ANOVA and Tukey’s post hoc test were done for each parameter at the end of week 12 with significance defined as P < 0.05. *P < 0.05 vs. CTL-ND rats; #P < 0.05 vs. CTL-D rats and $P <0.05 vs. HG-ND rats. There was no significant difference between testosterone and E2 levels of CTL-D and HG-ND, P>0.05. There was no significant difference between FSH and LH levels between studied groups, P>0.05

**Fig. 2 Effect of diabetes and hypogonadism on renal oxidative stress.**

Experiment I: Assessment of oxidant markers, (A) superoxide dismutase (SOD), (B) malondialdehyde (MDA), and (C) glutathione peroxidase (GPx) reaction in non-diabetic (ND) and diabetic (D) in control (CTL) and hypogonadal diabetic (HG) rats. Homogenate from renal HG-D group showed decrease in SOD and GPx levels but increase in MDA level compared with CTL-
ND and HG-ND groups. Values are mean ± S.D. analyzed by one-way ANOVA and Tukey's test and significance was defined as $P < 0.05$. *$P < 0.05$ vs. CTL-ND rats; #$P < 0.05$ vs. CTL-D rats and $\$P <0.05$ vs. HG-ND rats. There was no significant difference between assessed marker levels of CTL-ND and HG-ND, $P >0.05$. N=number of animals per group.

**Fig. 3 Effect of diabetes and hypogonadism n renal IL-1β, IL-6 and TNFα inflammatory levels.**

**Experiment I:** Assessment of inflammatory cytokines (A) interleukin 1β (IL-1β), (B) interleukin-6 (IL-6), and (C) tumor necrotic factor α (TNFα) reaction in non-diabetic (ND) and diabetic (D) control (CTL) and hypogonadal diabetic (HG) rats. Homogenate from renal HG-D group showed increase in inflammatory markers level compared with CTL-ND and HG-ND groups. Values are mean ± S.D. analyzed by one-way ANOVA and Tukey's test and significance was defined as $P < 0.05$. *$P < 0.05$ vs. CTL-ND rats; #$P < 0.05$ vs. CTL-D rats and $\$P <0.05$ vs. HG-ND rats. There was no significant difference between assessed marker levels of CTL-ND and HG-ND, $P >0.05$. N=number of animal per group.

**Fig. 4 Correlation between glycated hemoglobin (Hb-A1c) and renal inflammatory marker NF-κB expression in hypogonadal rats.** Experiment II in HG rats: Real-time PCR assessment of NF-κB renal expression shows the correlation between Hb-A1c and NF-κB expression level in the studied groups: control hypogonadal-diabetic, CTL HG-D (circle, blue), HG-D/letrozole, HG-D/LET (square, red), HG-diabetes/valsartan, HG-D/VAL (triangle, green) and HG-D/LET+VAL (circle, black). Data were fitted with a Pearson correlation (P and R2 values are shown). $P< 0.05$ was defined as significant. Number of animal per group is eight.
**Fig. 5 Renal angiotensin II (ANG II) level in diabetic and non-diabetic hypogonadal rats.**

Experiment II in HG rats: assessment of renal ANG II level showed inhibition in response to letrozole and valsartan. CTL; control without treatment, Hypogonadism, HG; non-diabetic, ND; diabetic, D; LET, letrozole (0.5 mg/Kg/day); VAL, valsartan (30 mg/Kg/day). ANG II values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA and Tukey's test. Significance was defined as P < 0.05. *P < 0.05 vs. corresponding values of control HG-D without treatment (black square), #P < 0.05 vs. HG-D/ LET, $P < 0.05 vs. HG-D/ VAL. §P<0.05 HG-ND (white square) vs. HG-D (black square) of the same group in CTL and treated groups. P> 0.05; NS, non-significant.

**Fig. 6 Effect of letrozole and valsartan on renal oxidative stress in hypogonadal diabetic rats.**

Experiment II, assessment of oxidant markers (A) superoxide dismutase (SOD), (B) malondialdehyde (MDA), and (C) glutathione peroxidase (GPx) reaction in non-diabetic (ND) and diabetic (D) hypogonadal (HG) rats without treatment (CTL) and treated with letrozole (LET), valsartan (VAL) or both (LET + VAL). Letrozole and valsartan improved oxidative stress markers in HG-D rats. LET, and VAL showed protecting effect against (HG-D). Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA and Tukey's test and significance was defined as P < 0.05. *P< 0.05 vs. corresponding values of control HG-D without treatment (black square), #P < 0.05 vs. HG-D/ LET, $P < 0.05 vs. HG-D/ VAL. §P<0.05 HG-ND (white square) vs. HG-D (black square) of the same group in CTL and treated groups. NS, non-significant.
**Fig. 7 Renal IL-1β, IL-6 and TNFα inflammatory levels in hypogonadal diabetic rats.**

Experiment II, assessment of inflammatory cytokines (A) interleukin 1β (IL-1β), (B) interleukin-6 (IL-6), and (C) tumor necrotic factor α (TNFα) reaction in hypogonadal (HG) non-diabetic (ND) and diabetic (D) rats without treatment (CTL) and treated with letrozole (LET), valsartan (VAL) or both (LET + VAL). LET, and VAL showed protecting effect against (HG-D). Values are mean ± S.D. (n= 6-8), analyzed by one-way ANOVA and Tukey's test and significance was defined as P < 0.05. Letrozole and valsartan improved inflammatory level in HG rats. *P< 0.05 vs. corresponding values of HG-D control (without treatment), #P < 0.05 vs. HG-D/ LET, $P < 0.05 vs. HG-D/ VAL. §P<0.05 HG-ND vs. HG-D of the same group in CTL and treated groups. NS, non-significant.

**Fig. 8 Kidney microscopic photograph of hypogonadal non-diabetic and diabetic rats.**

Diabetic nephropathy lesions by light microscopy. Photomicrographs of hematoxylin and eosin (H&E; Fig. 8A), periodic acid-Schiff (PAS; Fig. 8B) and Masson’s trichrome-stained sections (Fig. 8C) of the renal cortex on week 12 of experiment II. Glomerulus and tubules are shown at original magnification (400×) magnification; (I) control hypogonadal non-diabetic rats (CTL HG-ND), (II) control hypogonadal diabetic, CTL HG-D, (III) HG-D/ letrozole (LET), (IV) HG-D/ valsartan (VAL), and (V) HG-D/ LET + VAL. A, H&E stain shows normal glomeruli (G) and tubule (T) in (I) CTL HG-ND and patent glomerular capillary (red arrows), but (II) glomerular congestion and hypertrophy (GH) and inflammatory cell infiltrate (yellow arrows) and narrow Bowman’s space (black arrows) in CTL HG-D group. LET and VAL ameliorated these changes. B, PAS stain shows in (I) normal glomerulus (G) in CTL-HG group, open tubular lumen (T), thin glomerular wall (yellow arrow); (II) CTL HG-D group shows glomerular hypertrophy and diffuse glomerular hyaline sclerotic deposition (yellow asterisk), tubular obliteration, tubular vacuoles with dilated
lumen (orange arrows) and interstitial hyaline deposition with tubular thickening (white asterisk) are seen. LET shows moderate changes, and VAL shows mild changes. Combination (LET + VAL) group shows less injury to glomeruli and tubules. C, Masson's trichrome stain in (II) CTL HG-D, shows capsular, intraglomerular and intestinal fibrosis (yellow arrows) that decreased with LET and VAL treatment. Scale bars = 20 µm.

**Fig. 9 Reno-protective effect of letrozole and valsartan against diabetes associated collagen IV in hypogonadal rats.** Experiment II: Correlation between collagen IV deposition and a histopathological score of glomerular sclerosis. (A) control hypogonadal-diabetic, CTL HG-D, (B) HG-D/letrozole, HG-D/LET, (C) HG-diabetes/valsartan, HG-D/VAL, (D) HG-D/LET+VAL. Data were fitted with a Pearson correlation (P and R2 values are shown). Histopathological score, collagen IV values are mean ± S.D. (n= 8), analyzed by one-way ANOVA and Tukey's test and significance was defined as P < 0.05. #P< 0.05 vs. CTL HG-D, $P < 0.05 vs. HG-D/LET+VAL.

**Fig. 10 Reno-protective effect of letrozole and valsartan against diabetes associated TGFβ-1 deposition in hypogonadal rats.** Experiment II: Correlation between TGFβ 1 and a histopathological score of glomerular sclerosis. (A) control hypogonadal-diabetic, HG-D, (B) HG-D/letrozole, HG-D/LET, (C) HG-diabetes/valsartan, HG-D/VAL, (D) HG-D/LET+VAL. Data were fitted with a Pearson correlation (P and R2 values are shown). Histopathological score, TGFβ-1 values are mean ± S.D. (n= 8), analyzed by one-way ANOVA and Tukey's test and significance was defined as P < 0.05. #P< 0.05 vs. HG-D, $P < 0.05 vs. HG-D/LET+VAL.
Fig. 1

A

Testosterone (ng/ml)

CTL-ND (n=6)  CTL-D (n=6)  HG-ND (n=6)  HG-D (n=6)

B

E2 (pg/mL)

CTL-ND (n=6)  CTL-D (n=6)  HG-ND (n=6)  HG-D (n=6)

C

FSH (mIU/mL)

CTL-ND (n=6)  CTL-D (n=6)  HG-ND (n=6)  HG-D (n=6)

D

LH (mIU/mL)

CTL-ND (n=6)  CTL-D (n=6)  HG-ND (n=6)  HG-D (n=6)
Fig. 2

A

SOD (U/g protein)

CTL-ND  n=6

CTL-D  n=6

HG-ND  n=6

HG-D  n=6

B

MDA (nmol/ml g protein)

CTL-ND  n=6

CTL-D  n=6

HG-ND  n=6

HG-D  n=6

C

GPx (U/g protein)

CTL-ND  n=6

CTL-D  n=6

HG-ND  n=6

HG-D  n=6

81x65mm (300 x 300 DPI)
Fig. 3

A

B

C

81x65mm (300 x 300 DPI)
Fig. 4

Relative NF-kB expression

Glycated hemoglobin (Hb)A1c

- CTL HG-D
  - \( R^2 = 0.80; P < 0.05 \)
- HG-D / LET
  - \( R^2 = 0.92; P < 0.001 \)
- HG-D / VAL
  - \( R^2 = 0.96; P < 0.0001 \)
- HG-D / VAL + LET
  - \( R^2 = 0.88; P < 0.05 \)
Fig. 5

Angiotensin II (pg/ml protein)

- **HG-ND**
- **HG-D**

- **CTL**
- **LET**
- **VAL**
- **LET+ VAL**

n = 6

$\$ ns

* *# $
**Fig. 6**

**A**

- HG-ND: 
- HG-D:

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**B**

- HG-ND: 
- HG-D:

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**C**

- HG-ND: 
- HG-D:

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81x65mm (300 x 300 DPI)
Fig. 7

A

B

C

81x65mm (300 x 300 DPI)
Fig. 8A

I. CTL HG-KD

II. CTL HG-D

III. HG-DI LET

IV. HG-DI VAL

V. HG-DI LET VAL

81x65mm (300 x 300 DPI)
**Fig. 9**

A. CTL HG-D

- **Collagen IV (ng/ml protein)**
  - **R² = 0.84, P = 0.001**

- **Histopathological (HP) score**
  - **Collagen 18.99±3.23**
  - **HP Score 2.25±0.92**

B. HG-D/LET

- **Collagen IV (ng/ml protein)**
  - **R² = 0.80, P = 0.003**

- **Histopathological (HP) score**
  - **Collagen 10.30±2.03**
  - **HP Score 0.75±0.71**

C. HG-D/VAL

- **Collagen IV (ng/ml protein)**
  - **R² = 0.77, P = 0.004**

- **Histopathological (HP) score**
  - **Collagen 8.44±1.53**
  - **HP Score 1.90±1.01**

D. HG-D/LET+VAL

- **Collagen IV (ng/ml protein)**
  - **R² = 0.91, P = 0.0002**

- **Histopathological (HP) score**
  - **Collagen 4.70±1.40**
  - **HP Score 0.63±0.74**
A  Fig. 10

CTL HG-D
$R^2=0.71, P=0.008$

TGF-$eta$ $21.202.70^{\pm}31.66$
HP Score: 2.25$^{\pm}0.89$

B

HG-D/LET:
$R^2=0.77, P=0.004$

TGF-$eta$ $1.174.80^{\pm}18.1$
HP Score: 0.75$^{\pm}0.71$

C

HG-D/VAL:
$R^2=0.90, P=0.0003$

TGF-$eta$ $1.160.20^{\pm}15.37$
HP Score: 1.00$^{\pm}1.01$

D

HG-D/LET+VAL:
$R^2=0.83, P=0.002$

TGF-$eta$ $1.118.90^{\pm}16.30$
HP Score: 0.63$^{\pm}0.74$

81x65mm (300 x 300 DPI)