Dose-dependent effects of dihydrotestosterone in the streptozotocin-induced diabetic rat kidney

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ABSTRACT

We have recently reported that castration exacerbates albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis associated with diabetic renal disease. The aim of the present study was to examine if these effects of castration can be attenuated with dihydrotestosterone (DHT) supplementation. The study was performed in castrated male Sprague-Dawley, streptozotocin (STZ)-induced diabetic rats treated with 0 mg/day DHT (DHT$_0$), 0.75 mg/day DHT (DHT$_{0.75}$) or 2.0 mg/day DHT (DHT$_{2.0}$) for 14 weeks. Treatment with 0.75 mg/day DHT attenuated castration-associated increases in urine albumin excretion (DHT$_0$, 81.2±18.1; DHT$_{0.75}$, 26.57±5.8 mg/day; P<0.05), glomerulosclerosis (DHT$_0$, 1.1±0.79; DHT$_{0.75}$, 0.43±0.043 AU; P<0.001), tubulointerstitial fibrosis (DHT$_0$, 1.3±0.12; DHT$_{0.75}$, 1.1±0.096 AU; P<0.05), collagen type IV (DHT$_0$, 3.2±0.11; DHT$_{0.75}$, 2.1±0.070 ROD; P<0.01), transforming growth factor-beta (DHT$_0$, 3.2±0.16; DHT$_{0.75}$, 2.1±0.060 ROD; P<0.01) and IL-6 (DHT$_0$, 0.37±0.011; DHT$_{0.75}$, 0.27±0.014 ROD; P<0.05) protein expression and reduced CD68-positive cell abundance (DHT$_0$, 17±0.86; DHT$_{0.75}$, 4.4±0.55 cells/mm$^2$; P<0.001). In contrast, treatment with 2.0 mg/day DHT exacerbated all these parameters. These data suggest that the detrimental effects of castration in the diabetic kidney can be attenuated with low doses of DHT, while high doses augment the adverse effects of castration, and these effects appear to be influenced by estradiol. We conclude that the effects of DHT are dose-dependent but caution should be taken when DHT supplementation is considered in the treatment of diabetic renal disease.
INTRODUCTION

Low testosterone levels are commonly observed in men with both type 1 (7, 16) and type 2 diabetes with or without renal complications (5, 7). Studies have shown that there is a direct correlation between erectile dysfunction characterized by hypotestosteronemia and renal failure in men with either type 1 (11) or type 2 diabetes (2, 3). Experimental models of diabetic renal disease also exhibit low testosterone levels (24, 28). These observations strongly suggest that restoring testosterone levels to those observed in non-diabetics may actually prevent the development of renal disease associated with diabetes. However, little is known about the effects of testosterone in the diabetic kidney.

Studies in experimental models of non-diabetic renal disease, including hypertension-induced kidney injury (1, 6, 9, 10), ischemia/reperfusion-induced kidney injury (12, 20) and autosomal dominant polycystic kidney disease (19) have shown that castration or androgen receptor antagonism attenuate the development of renal injury. In contrast, our recent study in the STZ-induced diabetic rat has shown that castration exacerbates the severity of renal injury (28). These findings indicate that while in non-diabetic renal disease absence of testosterone is renoprotective, in the setting of diabetes, absence of testosterone appears to have an opposite effect and accelerates renal disease development. These observations further support the notion that treatment with testosterone may in fact be renoprotective in the diabetic kidney. Thus, the aim of the present study was to examine the effects of treatment with testosterone in the streptozotocin-induced rat model of diabetic renal disease. Furthermore, the aim of the study was to examine some of the mechanisms by which testosterone may exert its actions in the diabetic kidney.
MATERIALS AND METHODS

Animals. Castrated Sprague Dawley rats (12 weeks of age) were purchased from Harlan (Madison, WI) and maintained on standard rat chow and tap water ad libitum. The animals were rendered diabetic by a single intraperitoneal (ip) injection of 55 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO) in 0.1 M citrate buffer (pH 4.5) after an overnight fast. All animals were injected with insulin every 3 days (2-4 U, Lantus, Aventis Pharmaceuticals Inc., Kansas City, MO) to maintain blood glucose levels between 300-450 mg/dl, promote weight gain and prevent mortality. Throughout the treatment period (14 weeks), the animals were placed in metabolic cages for 24 h for collection of urine for analysis of urine albumin concentration and urine output. After 14 weeks of treatment, the animals were anesthetized with sodium pentobarbital (40 mg/kg ip) and their femoral vessels catheterized. Systemic blood pressure was monitored electronically using Cardiomax-II (Columbus Instruments, Columbus, OH) blood pressure analyzer. Following blood pressure measurements, the animals were weighed for measurement of sex hormone levels. The kidneys were weighed and then either snap frozen in liquid nitrogen for protein analysis or immersion fixed with HistoCHOICE (Amresco, Solon, OH) for immunohistochemical analysis. All protocols complied with the guidelines recommended by the National Institutes of Health and approved by the Georgetown University, as well the University of Mississippi Animal Care and Use Committee.

DHT treatment and measurement of plasma sex hormones. Two days after the induction of diabetes, the animals were anesthetized with 2% isofluorane and were implanted subcutaneously in their backs with continuous release pellets (Innovative Research, Sarasota,
The dose of DHT was chosen based on previous studies examining the effects of DHT treatment on renal injury (10). Plasma testosterone and estradiol (Assay Designs, Ann Arbor, MI) and DHT (Alpha Diagnostics, San Antonio, TX) levels were measured by ELISA, according to the manufacturer’s protocol, but with modified standard curves to fit the linear range of expected hormone levels.

**Urine albumin excretion (UAE).** Urine albumin concentration was measured using the Nephrat II albumin kit (Exocell, Inc., Philadelphia PA) according to the manufacturer’s protocol. The rate of UAE was calculated based on the measured concentrations and 24h urine output.

**Glomerulosclerosis and tubulointerstitial fibrosis.** The index of glomerulosclerosis (GSI) and tubulointerstitial fibrosis (TIFI) was assessed in PAS and Masson’s trichrome-stained paraffin sections, respectively, using a semi-quantitative method as previously described (15).

**Immunohistochemistry.** Immunolocalization of nestin, podocin, collagen type IV, TGF-β, CD68 and TNF-α was carried out as previously described (28) using the following antibodies: nestin (1:200, Millipore, Billerica, MA), podocin (1:400, Abcam, Cambridge, MA), collagen type IV (1:400, Southern Biotech, Birmingham, AL), TGF-β (1:400, R&D Systems, Minneapolis, MN), CD68 (1:600, Serotec, Oxford, UK) and TNF-α (1:200, Santa Cruz Biotech, Santa Cruz, CA). Macrophage number was assessed by counting the number of CD68-positive cells in 20 different fields in three sections per animal from each group and
expressed per mm$^2$. Nestin and podocin immunoexpression was assessed in twenty
randomly selected glomeruli per section and quantitated using image analysis software (NIS-
Elements, Ver. 2.32; Nikon Instruments, Melville, NY). The data are expressed as percentage
of area stained for nestin or podocin per glomerulus.

Western blotting. Expression of collagen type IV, TGF-β, TNF-α and IL-6 protein was carried
out as previously described (28) using the following antibodies: collagen type IV (1:1,000, Chemicon), TGF-β (1:500, R&D Systems), TNF-α (1:1,000, Santa Cruz), IL-6 (1:2,000, Santa
Cruz). The densities of specific bands were normalized to the total amount of protein loaded
in each well following densitometric analysis of gels stained with either Coomassie blue (for
collagen type IV) or β-actin (1:1,000, Santa Cruz) for all other proteins. The densities of
specific bands were quantitated by densitometry using the Scion Image beta (version 4.02)
software.

TUNEL staining. Apoptotic cell death was detected in paraffin sections using the in situ cell
death detection kit, POD (Roche Diagnostics, Indianapolis, IN) according to the
manufacturers protocol.

Statistical analysis. Data are expressed as mean±SEM and were analyzed using a one-way
analysis of variance (ANOVA). Post hoc comparisons were performed with a Tukey’s test
(Prism 4, Graph Pad Software, San Diego, CA). Significance was accepted at $P < 0.05$.

RESULTS

Metabolic parameters and blood pressure. Blood glucose, body weight, body/kidney weight
and food intake were similar in all the treatment groups (Table 1). Interestingly, the DHT_{2.0} rats exhibited a 10% and 17% reduction in urine output compared with DHT_{0} and DHT_{0.75} rats, respectively (Table 1), despite no differences in water intake (Table 1). No differences in mean arterial pressure (MAP) were observed between the treatment groups (Table 1).

**UAE.** The DHT_{0.75} animals exhibited a 67% decrease in UAE compared with DHT_{0} animals, while UAE in DHT_{2.0} increased by 145% compared with DHT_{0} and by 651% compared with DHT_{0.75} animals (Table 1). No differences in mean arterial pressure were observed between any of the treatment groups (data not shown).

**Sex hormone levels.** (Table 1). Plasma DHT levels increased by 268% in DHT_{0.75} compared with DHT_{0} animals (Table 1), while no differences in plasma testosterone levels were observed between these two treatment groups. Both DHT and testosterone levels increased by 653% and 198%, respectively in DHT_{2.0} compared with DHT_{0} animals and by 105% and 89%, respectively compared with DHT_{0.75} animals (Table 1). No differences in plasma estradiol levels were observed between the DHT_{0} and DHT_{0.75} groups, while there was a 35% increase in DHT_{2.0} compared with both DHT_{0} and DHT_{0.75} animals (Table 1).

**Glomerulosclerosis and tubulointerstitial fibrosis.** As we have previously reported, the DHT_{0} animals exhibited prominent glomerular and tubulointerstitial injury characterized by mesangial expansion, accumulation of extracellular matrix (ECM) proteins and presence of inflammatory cells (Fig. 1A and 1B). These changes were largely attenuated in the DHT_{0.75} group, while DHT_{2.0} animals exhibited even more pronounced renal pathology than the DHT_{0}
animals (Fig. 1A and 1B). The semi-quantitative analysis of the degree of renal pathology showed a 61% decrease in glomerulosclerosis (Fig. 1C) and a more modest 15% decrease in tubulointerstitial fibrosis (Fig. 1D) in DHT0.75 compared with D0 animals. In contrast, the DHT2.0 animals exhibited a 36% increase in glomerulosclerosis (Fig. 1C) and 38% increase in tubulointerstitial fibrosis (Fig. 1D) compared with the DHT0 group. Interestingly, in addition to the usual characteristics of glomerulosclerosis, microaneurysms were also a common feature in the DHT2.0 group (Fig. 1A).

Podocyte markers. Nestin, an intermediate filament and the slit diaphragm protein podocin are molecular markers of podocytes (8, 25). Nestin and podocin-positive cells, identifying podocytes, were observed throughout the glomerular tuft in all the treatment groups (Fig. 2A and 2B). However, the immunostaining in the DHT0 and DHT2.0 groups was less prominent. Quantitative analysis showed a 23% and 33% decrease, respectively, in the percent area of glomerular tuft occupied by nestin and podocin-positive cells in the DHT2.0 compared with the DHT0 group (Fig. 2C and 2D).

Collagen type IV protein expression. In the DHT0 renal cortex, collagen type IV was immunolocalized to basement membranes of proximal and distal tubules and expanded mesangial areas in the glomerulus (Fig. 3A). DHT0.75 was associated with an overall decrease, while DHT2.0 was associated with an increase in the intensity of immunostaining compared with either DHT0 and DHT0.75 (Fig. 3A). Quantitative analysis of collagen type IV protein expression by Western blotting confirmed the immunohistochemical findings. Collagen type IV protein expression in the DHT0.75 group decreased by 34% compared with
DHT\(_0\) animals (Fig. 3B), while it increased by 34% in DHT\(_{2.0}\) compared with DHT\(_0\) animals (Fig. 3B).

**Inflammatory markers.** TGF-\(\beta\) immunoexpression was evident predominantly in the glomerular mesangial areas and distal tubules and to a lesser extent in the proximal tubules (Fig. 4A). The overall intensity of immunostaining was decreased in the DHT\(_{0.75}\) group, while it increased in the DHT\(_{2.0}\) compared with the DHT\(_{0.75}\) group (Fig. 4A). Western blot analysis showed a 34% decrease in TGF-\(\beta\) protein expression in the DHT\(_{0.75}\) compared with DHT\(_0\), while the DHT\(_{2.0}\) animals showed a 38% increase in TGF-\(\beta\) protein expression compared with DHT\(_0\) (Fig. 4B).

CD68-positive cells, indicating the presence of macrophages, were prominent in the glomeruli and tubulointerstitial areas in the kidneys of DHT\(_0\) animals (Fig. 5A). In the DHT\(_{0.75}\) group, there was a 74% decrease in the abundance of CD68-positive cells, while DHT\(_{2.0}\) animals showed a 17% increase in CD68-positive cell abundance compared with DHT\(_0\) (Fig. 5B).

TNF-\(\alpha\) was immunolocalized to the thick ascending limb of the loops of Henle in the outer stripe of the outer medulla in all the treatment groups (Fig. 6A); however, the intensity of immunostaining was greater in the DHT\(_0\) and DHT\(_{2.0}\) groups compared with the DHT\(_{0.75}\) group. While no differences in the expression of TNF-\(\alpha\) protein was observed between the DHT\(_0\) and DHT\(_{0.75}\) groups (Fig. 6B) as measured by Western blotting, there was a 83% and 72% increase in the expression of TNF-\(\alpha\) protein in the DHT\(_{2.0}\) group compared with DHT\(_0\) and DHT\(_{0.75}\) groups, respectively (Fig. 6B). Western blotting also revealed a 27% decrease in IL-6 protein expression in the DHT\(_{0.75}\) compared with DHT\(_0\) group, but a 37% and 74%
increase in IL-6 protein expression in the DHT_{2.0} compared with the DHT_{0} and DHT_{0.75} group, respectively (Fig. 6C). Note: we have not been able to detect IL-6 by immunohistochemistry.

*TUNEL staining.* TUNEL-positive cells, indicating apoptosis, were localized predominantly in the glomerulus (most likely podocytes based on morphology) in the DHT_{0} animals (Fig. 7A). Very few TUNEL-positive cells were apparent in the DHT_{0.75} animals, while in the DHT_{2.0} animals, TUNEL-positive cells were highly abundant in both the glomerulus and surrounding tubulointerstitium (Fig 7A). Quantitative analysis revealed an 87% decrease in of TUNEL-positive cell abundance in DHT_{0.75} compared with DHT_{0} animals (Fig. 7B). TUNEL-positive cell abundance increased by 85% and 1288% in DHT_{2.0} compared with DHT_{0} and DHT_{0.75} animals, respectively (Fig. 7B).

**DISCUSSION**

The present study demonstrates that while a lower dose of DHT attenuates the development of albuminuria, glomerulosclerosis and tubulointerstitial fibrosis associated with diabetes, a higher dose of DHT exacerbates renal injury in castrated diabetic rats. These observations indicate that DHT may play an important role in the pathophysiology of diabetic renal disease, but that its effects are dose-dependent.

The findings of the present study confirm our previous report that unlike in experimental models of non-diabetic renal disease in which castration, and thus absence of endogenous testosterone is renoprotective, in diabetes, castration exacerbates renal disease (28). Specifically, castration is associated with an increase in urine albumin excretion, glomerulosclerosis and tubulointerstitial fibrosis via increasing the expression of TGF-β,
collagen type I and type IV, increasing the abundance of activated macrophages and decreasing the expression and activity of matrix metalloproteinases (28). Based on these observations, we concluded that the absence of testosterone is detrimental to the diabetic kidney. These observations provided a rationale for the present study, which examined the potential protective effects testosterone (i.e. its more biologically active metabolite, DHT) treatment in the diabetic kidney.

The present study shows that a low dose of DHT (0.75 mg/day) attenuates much of the castration-associated renal injury. Specifically, DHT$_{0.75}$ reduced albuminuria, glomerulosclerosis and tubulointerstitial fibrosis via reducing collagen type IV, TGF-β and IL-6 protein expression and also by reducing CD68-positive cell density and apoptosis. Based on these observations that the absence of androgens was detrimental, the beneficial effects of DHT observed in the present study were not that surprising. DHT has also been shown to have neuroprotective effects in an experimental model of diabetic neuropathy (22). However, these observations are in contrast to the majority of studies in experimental models of non-diabetic renal disease that show detrimental effects of testosterone treatment (1, 6, 10). To the best of our knowledge, there is only one report in the STZ-induced diabetic rat, albeit of pre-pubertal age, showing that treatment with testosterone promotes tubular damage and albuminuria (24). However, no study to date has examined the renal effects of DHT treatment in adult STZ-induced diabetic rats. In contrast to DHT$_{0.75}$, treatment with a higher dose of DHT (2.0 mg/day) exacerbated albuminuria, glomerulosclerosis and tubulointerstitial fibrosis via increasing collagen type IV, TGF-β and IL-6 protein expression and by increasing CD68-positive cell density and apoptosis. Furthermore, DHT$_{2.0}$ exacerbated TNF-α protein expression and reduced the volume density of podocytes in glomeruli. These observations
are more in line with the previous studies in experimental models of non-diabetic renal
disease showing adverse effects of testosterone (1, 6, 10, 24). Notably, these studies
suggested that the effects of testosterone or DHT on the kidney are mainly related to their
hypertensive effects (1, 6, 10). However, no differences in blood pressure were observed in
any of the treatment groups in the present study, suggesting that in the diabetic kidney, the
effects of DHT are most likely mediated via a more direct mechanism. Indeed, we show that
DHT$_{2.0}$ promotes extracellular matrix deposition, proximal tubular and podocyte cell death and
inflammation. In the STZ-induced diabetic rat, testosterone has been shown to promote
tubulointerstitial fibrosis by increasing the expression of TGF-β (24). In a proximal human
tubule cell line (HK-2 cells) cultured under high glucose conditions, testosterone promotes
apoptosis via caspase activation (26). Others have shown that testosterone increases TNF-α
production and proapoptotic and profibrotic signaling pathways during renal obstruction,
resulting in increased apoptotic cell death, tubulointerstitial fibrosis, and renal dysfunction
(18). Our study supports these reports of pro-inflammatory effects of DHT, as treatment with
DHT$_{2.0}$ increased renal expression of TNF-α and IL-6. Diabetic renal disease has been
shown to be associated with the loss of podocytes (17, 30). Our studies show a decrease in
the expression of podocyte markers in the STZ-induced diabetic rat kidney, nestin and
podocin (8, 25), suggesting that diabetes, especially after treatment with DHT$_{2.0}$, is
associated with reduced volume density of podocytes in glomeruli. Although the precise
mechanisms for the loss of podocytes in diabetic glomeruli are as of yet unknown, apoptosis
has been suggested to play a role (27). In our study, apoptosis was most prominent in
podocytes, especially after DHT$_{2.0}$ treatment, suggesting that one of the mechanisms by
which DHT$_{2.0}$ promotes diabetic renal disease, in addition to promoting inflammation and extracellular matrix deposition, may be via podocyte apoptosis.

Observations from the present study point to a dual and dose-specific effect of DHT in the diabetic kidney: while low doses of DHT are renoprotective, higher doses are damaging. The question remains, what are the determinants of these seemingly opposing effects of DHT? There are several potential explanations for this apparent paradox, including dose-dependent expression and activation of androgen receptor-interacting transcriptional coactivator proteins; however, the most likely explanation is the indirect effect of estradiol rather than the direct effects of DHT. The present study confirms previous observations from this and other laboratories on the appreciable amounts of circulating estradiol in castrated diabetic (24, 28) or non-diabetic (10) rats. These findings suggest that estradiol, following castration, may originate from organs other than the testes, such as the adrenal gland (23) or even the kidney itself (21). Indeed, unpublished observations from our laboratory show expression of aromatase (an enzyme responsible for conversion of testosterone into estradiol), predominantly at sites of renal injury in the diabetic kidney. Interestingly, the DHT$_{2.0}$ group that was associated with the greatest degree of renal damage also exhibited the highest levels of estradiol. Based on these findings, we propose that renal estradiol synthesis may be triggered in response to injury and that once synthesized, it may exert detrimental effects in the male diabetic kidney. This is in contrast to the renoprotective effects of estradiol observed in diabetic female rats (4, 13-15). However, how local production of estradiol, either in the kidney or other target organs results in increased circulating levels of estradiol warrants further investigation. Further supporting the notion that the opposing effects of DHT observed in the present study may be related to estradiol rather than DHT directly, is the fact that the
levels of circulating DHT achieved by the treatments do not directly correlate with the degree of renal injury. No DHT (as in castration) as well as high levels of DHT (2.0 mg/day) were both associated with pronounced renal injury, while the injury was prevented with intermediate levels of DHT (0.75 mg/day). Interestingly, intact diabetic animals that are characterized by renal injury milder than either the DHT$_0$ or DHT$_{2.0}$ exhibit circulating DHT levels comparable to that of DHT$_{2.0}$ (data not shown). Similarly, there is no direct correlation between testosterone levels and the degree of renal injury. In fact, there appears to be a dose-dependent increase in testosterone levels with DHT treatment; however, this is most likely a result of cross-contamination with DHT in the assays used to measure hormone levels. These observations suggest that plasma levels of estradiol, rather than DHT or testosterone, may be a better predictor of the degree of renal disease in diabetes. Indeed, a recent clinical study has reported that alongside reduced testosterone levels, men with chronic renal disease, most of which are diabetic, also exhibit elevated estradiol levels that correlate with the decline in renal function (29). Overall, while observations from our study suggest that treatment with DHT, at least at low levels are renoprotective, either directly or indirectly via interaction with estradiol, caution should be taken in extrapolating the potential beneficial effects of DHT treatment, even at low doses in humans with diabetic renal disease.

In summary, the present study demonstrates that the effects of DHT in the diabetic kidney may be dual and dose-dependent and that these effects may influenced by estradiol. These studies underscore the importance of sex hormones in the pathophysiology of diabetic renal disease and the importance of further examining the mechanisms by which sex hormones regulate renal function in the diabetic kidney.
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DISCLOSURES

All authors have no relationship with companies that may have a financial interest in the information contained in the present investigation.

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Fig. 1. Renal pathology. A. PAS-stained sections of the renal cortex. B. Masson’s trichrome-stained sections of the renal cortex. C. Glomerulosclerosis index (GSI) D. Tubulointerstitial fibrosis index (TIFI). Abbreviations and symbols: glomerulus (g); proximal tubule (pt); distal tubule (dt); microaneurysms (ma); mesangial cell (arrow head); area of ECM protein deposits (outlined in black). Original magnification X400. Data are expressed as mean±SEM.

Fig. 2. Nestin and podocin immunoexpression. A. Nestin immunolocalization. B. Podocin immunolocalization. Abbreviations: glomerulus (g). Original magnification X400. C. Quantitative analysis of podocin immunoexpression. Data are expressed as mean±SEM.

Fig. 3. Collagen type IV protein expression. A. Collagen type IV immunolocalization. Abbreviations: glomerulus (g); proximal tubule (pt); distal tubule (dt). Original magnification X400. B. Collagen type IV protein expression. Top panel, representative immunoblot of collagen type IV protein expression. Bottom panel, densitometric scans in relative optical density (ROD) expressed as a ratio of collagen type IV/Coomassie blue. Data are expressed as mean±SEM.

Fig. 4. TGF-β protein expression. A. TGF-β immunolocalization. Abbreviations and symbols: glomerulus (g); proximal tubule (pt); distal tubule (dt); mesangial cell (arrow head). Original magnification X400. B. TGF-β protein expression. Top panel, representative immunoblot of TGF-β protein expression. Bottom panel, densitometric scans in relative optical density (ROD) expressed as a ratio of TGF-β/β-actin. Data are expressed as mean±SEM.
Fig. 5. CD68 abundance. A. CD68 immunolocalization. Abbreviations and symbols: glomerulus (g); proximal tubule (pt); distal tubule (dt); CD68-positive cell (arrow head). Original magnification X400. B. Quantitative analysis of CD68-positive cell abundance. Data are expressed as mean±SEM.

Fig. 6. TNF-α and IL-6 expression. A. TNF-α immunolocalization. Abbreviations and symbols: proximal tubule (pt); loop of Henle (lh), inflammatory cells (arrow head). Original magnification X400. B. TNF-α protein expression. Top panel, representative immunoblot of TNF-α protein expression. Bottom panel, densitometric scans in relative optical density (ROD) expressed as a ratio of TNF-α/β-actin. C. IL-6 protein expression. Top panel, representative immunoblot of IL-6 protein expression. Bottom panel, densitometric scans in relative optical density (ROD) expressed as a ratio of IL-6/β-actin. Data are expressed as mean±SEM.

Fig. 7. TUNEL staining. A. TUNEL-positive cell localization. B. Quantitative analysis of TUNEL-positive cell abundance. Abbreviations and symbols: glomerulus (g); proximal tubule (pt). Data are expressed as mean±SEM.
### Table 1. Metabolic and renal parameters

<table>
<thead>
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<th>Parameters</th>
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<td>Blood glucose (mg/dl)</td>
<td>350±13</td>
<td>373±15</td>
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<td>Body weight (g)</td>
<td>281±13</td>
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<td>Water intake (ml/day)</td>
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<td>Urine output (ml/day)</td>
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<td>130±6</td>
<td>108±14 †</td>
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<td>UAE (mg/day)</td>
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<td>MAP (mmHg)</td>
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Data are expressed as mean±SEM. n=10/group. Statistical significance was accepted at $P<0.05$.

* $P<0.05$ vs DHT₀, ** $P<0.001$ vs DHT₀

† $P<0.05$ vs DHT₀.₇₅, †† $P<0.01$ vs DHT₀.₇₅

Abbreviations: DHT₀, diabetic castrated rat treated with 0 mg/day DHT

DHT₀.₇₅, diabetic castrated rat treated with 0.75 mg/day DHT

DHT₂.₀, diabetic castrated rat treated with 2.0 mg/day DHT

UAE, urine albumin excretion

MAP, mean arterial pressure

kw, kidney weight
Fig. 1

A

B

C

D

GSI (AU)

TIFI (AU)

DHT₀ DHT₀.₇₅ DHT₂.₀

0.0 0.5 1.0 1.5 2.0 2.5

P<0.001

P<0.001

P<0.001

P<0.05

P<0.01

P<0.01
Fig. 2

Nestin-positive cells (% glomerular area stained)

- D0
- D0.75
- D2.0

P < 0.05

Podocin-positive cells (% glomerular area stained)

- D0
- D0.75
- D2.0

P < 0.001
Fig. 3

Collagen IV protein/Coomassie blue (ROD)

DHT₀, DHT₀.₇₅, DHT₂.₀

P<0.01
P<0.001
Fig. 4

A

DHT_0  DHT_{0.75}  DHT_{2.0}

g  pt  g  pt  g

dt  dt  dt

B

TGF-β

β-actin

TGF_β protein/β-actin (ROD)

DHT_0  DHT_{0.75}  DHT_{2.0}

P<0.05  P<0.05  P<0.05
Fig. 5

A

DHT₀ DHT₀.₇₅ DHT₂.₀

B

Number CD68-positive cells/mm²

![Graph showing number of CD68-positive cells in different conditions: DHT₀, DHT₀.₇₅, and DHT₂.₀.](image)
Fig. 6

A

DHT\textsubscript{0} \hspace{1cm} DHT\textsubscript{0.75} \hspace{1cm} DHT\textsubscript{2.0}

lh \hspace{1cm} lh \hspace{1cm} lh

pt

B

\begin{align*}
&\text{TNF-\textalpha} \\
&\beta\text{-actin}
\end{align*}

\begin{align*}
\text{DHT}\textsubscript{0} & \quad \text{DHT}\textsubscript{0.75} & \quad \text{DHT}\textsubscript{2.0} \\
\hline
\text{TNF-\textalpha} & \text{ROD} & \text{ROD} & \text{ROD} \\
\beta\text{-actin} & \text{ROD} & \text{ROD} & \text{ROD}
\end{align*}

C

\begin{align*}
&\text{IL-6} \\
&\beta\text{-actin}
\end{align*}

\begin{align*}
\text{DHT}\textsubscript{0} & \quad \text{DHT}\textsubscript{0.75} & \quad \text{DHT}\textsubscript{2.0} \\
\hline
\text{IL-6} & \text{ROD} & \text{ROD} & \text{ROD} \\
\beta\text{-actin} & \text{ROD} & \text{ROD} & \text{ROD}
\end{align*}
Fig. 7

A

DHT₀  DHT₀.₇₅  DHT₂.₀

B

% TUNEL-positive cells/glomerulus

P<0.01  P<0.001  P<0.01