# Improved Histoarchitectural Changes with Angiotensin Receptor Blockers in Early Testicular and Cauda Toxicity in Rats

Mejora de los Cambios en la Histoarquitectura con Bloqueadores de Receptores de Angiotensina en la Toxicidad Precoz Testicular y de Cauda en Ratas

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**SUMMARY:** Reproductive dysfunction is a complication for many diseases and toxins. Its early diagnosis and treatment are immensely important. Here the morphological histoarchitecture changes in early testicular and cauda toxicity before and after treatment with angiotensin receptor blockers were evaluated. Low-grade testicular damage was induced using thioacetamide (TAA, 50 mg/kg/day) intraperitoneally for two weeks in rats. The rats were randomly divided into four groups (n = 8) treated daily orally for three weeks as follows: Normal control (distilled water), TAA (positive control), TAA+candesartan (0.2 mg/kg) and TAA+losartan (7.5 mg/kg). Serum testosterone and testicular malondialdehyde and glutathione were measured. The changes in histoarchitecture of testis and cauda epididymis were evaluated by hematoxylin and eosin for general structure, Masson's trichrome for collagen, periodic acid Schiff for basement membrane, and caspase-3 and proliferating cell nuclear antigen (PCNA) for immunohistochemical analysis. The TAA-rats showed decreases of serum testosterone and testicular glutathione, increases in testicular malondialdehyde, degenerative changes and apoptosis in germ cells, thickening of tubular basal lamina and increases in expression of caspase 3, and decreases in expression of PCNA. The ARBs (candesartan and losartan) significantly reversed these changes with non-significant differences in-between. Treatment with ARBs (candesartan and losartan) significantly reversed TAA-induced low-grade testicular and cauda toxicity in rats. This could be potentially useful for early treatment of male patients with occupational toxicant-induced reproductive dysfunction especially if they are using ARBs for other comorbidities.

KEY WORDS: Caspase-3; Masson; Proliferating cell nuclear antigen; Angiotensin receptor blockers.

#### INTRODUCTION

Male hypogonadism is a common problem in patients with liver cirrhosis which partially improves after liver transplantation (Foresta *et al.*, 2008). The underlying mechanism of such problem is complex and not well explained. Liver is involved in metabolism of sex hormones, thus pronounced changes in such metabolism, free serum testosterone level, and sex hormone-binding globulin level in blood could be possible causes (Nitsche *et al.*, 2014). Thioacetamide-induced liver damage was associated with testicular toxicity (Kang *et al.*, 2006). In testicular toxicity, use of histopathological measures is more sensitive parameter of testicular damage than use of testis weight and sperm counts and thus can detect early toxicity.

Proliferating cell nuclear antigen (PCNA) is a nuclear matrix protein necessary for several cell cycle pathways. Significant decreases in PCNA immunohistochemistry measured on formalin-fixed paraffin-embedded rat testes have been used to identify and quantify of the proliferation-related toxicity. Thus PCNA assay is potentially useful in vivo biomarker for detecting early testicular toxicity and for follow-up of compounds with low testicular toxicity (D'Andrea *et al.*, 2008). The block of effects of angiotensin II whether by angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) have been found to protect the testicular tissue against multiple insults (Alves-

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Pereira *et al.*, 2014). Both captopril and telmisartan reversed the cadmium-induced testicular damage with nonsignificant differences in-between. They increased serum testosterone level and the testicular reduced glutathione and decreased testicular MDA level and expression of testicular caspase-3 (Fouad & Jresat, 2013). ACEIs and ARBS are used to treat cardiovascular diseases like hypertension and heart failure, to prevent renal failure in cases of hypertension and/or diabetes, and to decrease the risk of stroke. Generally ARBs are more tolerated than ACEIs.

Taken together, this study was designed to evaluate the morphological changes in testicular histoarchitecture before and after treatment with angiotensin receptor blockers in early testicular toxicity induced by low dose thioacetamide in rats.

## MATERIAL AND METHOD

**Animals and Drugs.** The study was approved by the Institutional Research Ethics Committee. It agreed with the International guidelines for use of Laboratory animals. Sprague–Dawley male rats (200-250 g) were housed in cages at 22 °C room temperature with food and water ad libitum. Drugs and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Induction of low-grade testicular toxicity. Low grade testicular toxicity was induced in rats by injection of thioacetamide (TAA, 50 mg/kg) intraperitoneally once daily for two weeks. This small dose was used before, to induce low-grade hepatotoxicity. The rats were randomly divided into four groups (n = 8): negative control group (distilled water), TAA (positive control) group, candesartan cilexetil group (0.2 mg/kg/day) (Gaur & Kumar, 2011), and losartan potassium group (7.5 mg/kg/ day) (Croquet et al., 2002). Treatments were given orally for three weeks. At the end of treatment duration, blood was collected through the retroorbital plexus, centrifuged at 4000 rpm for 5 min, and serum was separated and kept at -80°C for biochemical measurements. Rats were sacrificed by cervical dislocation and the male system was removed as one block for preparation of the testicular homogenate, and histopathological and immunohistochemical examination. For histopathological and immunohistochemical examination the testis and cauda were fixed in 10 % neutral buffered formalin then processed for paraffin sections. Slides were stained by hematoxylin and eosin for general structure, periodic acid Schiff (PAS) for basement membrane, Masson trichrome

for collagen, and immunohistochemically for caspase-3 and proliferating cell nuclear antigen (PCNA). Photographs were taken for all groups and compared (Suvarna *et al.*, 2013).

Assay of serum testosterone. The serum testosterone level was measured by an ELISA kit (Sigma, MO, US) as per instructed by the manufacturer.

**Measurement of testicular MDA and GSH.** The testes were homogenized in ice-cold medium of 50 mM Tris-HCl (pH 7.4), the homogenate was centrifuged at 1000 xg for 10 min at 4 °C, the supernatant was used for measurement of MDA & GSH. For estimation of MDA, the testis homogenate was incubated in 10 % trichloroacetic acid and 0.67 % thiobarbituric acid (1 mL of each) for 30 min (Ohkawa *et al.*, 1979). The GSH was determined by the reduction of Elman's reagent (Ellman, 1959).

## Immunohistochemistry of testis and cauda

**Caspase-3.** The testicular tissue sections were dewaxed and rehydrated. Endogenous peroxidase was blocked using  $0.3 \ \% H_2O_2$  in methanol for 15 min. Then sections were incubated with primary diluted polyclonal antibodies for caspase-3 (Thermo Fisher Scientific Co., USA) for 30 min. and subsequently with biotinylated polyvalent secondary antibody (Thermo Fisher Scientific Co., USA) for 30 min. Metal Enhanced DAB Substrate Working Solution was added to the tissue followed by counterstaining with hematoxylin stain (Kim *et al.*, 2001; Elgawish & Abdelrazek, 2014).

**Proliferative cell nuclear antigen (PCNA).** After deparaffinization and rehydration steps, the testicular tissue sections were incubated in methanol containing 0.3 % H2O2 and normal goat serum for 15 minutes to decrease non-specific peroxidase reactions. Then, they were incubated for 30 minutes with a primary antibody against PCNA (Santa Cruz Biotech., Texas, US) and subsequently with goat anti-rat IgG as a secondary antibody (Sigma, MO, USA). After that, tissue sections were incubated with the peroxidase/anti-peroxidase complex for 90 min. Diaminobenzidine was used as chromogen followed by counterstaining with hemotoxylin and examination by a light microscope (Sternberger, 1986; Altay *et al.*, 2003; Dkhil *et al.*, 2016).

**Statistical analysis.** Data was expressed as means  $\pm$  SEM and analyzed using SPSS version 18. One-way ANOVA followed by Tukey's multiple comparison post-hoc test was used to assess differences between groups. P < 0.05 was considered to be statistically significant.

### RESULTS

**Serum testosterone and Testicular MDA and GSH.** TAA decreased serum testosterone level, increased testicular MDA level while decreased testicular GSH level compared with the normal control group. Both candesartan and losartan

significantly reversed these change with non-significant difference in-between (Table I).

**The histoarchitectural study.** Both candesartan and losartan protected against TAA-induced testicular and cauda toxicity as shown using the following stains: Hematoxylin and eosin (Figs. 1 and 2), Masson trichrome (Fig. 3), PAS (Fig. 4), Caspase-3 (Fig. 5), and PCNA (Fig. 6).



Fig. 1. Sections of rat testis stained by H&E photographed by low power and high power . (A) Normal Control group showing normal rounded seminiferous tubules (ST) with intact full germ cell layer (double headed arrows), and normal population of interstitial Leydig cells (white arrow). (B)TAA group showing degeneration and depletion of germ cell layer (black arrows). The interstitial tissue showed blood vessels with thick wall and Leydig cells looked smaller and degenerated (star). (C)TAA+Candesartan group: Most tubules looked normal with full thickness germ cell layers (double headed arrows). The interstitial spaces are normal and similar to NC (white stars). (D) TAA+Losartan group: Moderate improvement of TAA induced changes (1 & double arrows). Some tubules showed degenerative changes (2 & dotted arrows).



Fig 2. Sections of rat cauda epididymis stained by H&E low power and high power. (A) Normal Control group: The tubules (arrows) show variations in size and plan of cutting. They are separated by scanty connective tissue with few connective tissue cells (black stars). The tubules are lined by simple columnar ciliated epithelium. The cytoplasm is slightly basophilic and the nuclei are rounded, vesicular, and basal. The lumen is filled with mature sperms (white stars). (B) TAA group: There is increased interstitial tissue (black stars) with numerous inflammatory cells (dotted arrows). The epithelium showed degenerated cells and loss of apical cilia (white arrows). The lumen showed absence of mature sperms and is filled with acidophilic debris (white stars). (C) TAA+Candaesartan group: The tubules are still separated by wide spaces with fine fibrous tissue (black star) and few inflammatory cells (dotted arrow). The lumina contain mature sperms (black star). (D) TAA+Lorasartan group: The tubules are separated by interstitial spaces containing fibrous tissue (black stars). The lumina contain few mature sperm (white stars). The lining epithelium is normal with intact apical cilia (white arrows).

Group	Serum testo sterone	Testicular GSH	Testicular MDA
	(mmol/L)	(µg/g testis)	(mmol/mg protein)
Normal control	$3.26 \pm 0.14$	$57.63 \pm 0.90$	$16.91 \pm 0.45$
Thioacetamide	$1.71 \pm 0.11$	$38.59 \pm 1.55$	$29.61 \pm 0.63$
TAA+Candesartan	2.82±0.18 *	55.31 ± 1.33 *	$17.65 \pm 0.32$ *
TAA+Losartan	2.90 ± 0.14 *	53.75 ± 1.09 *	$18.14 \pm 0.38$ *
Data was expressed as mean $\pm$ SEM. *: P < 0.05 vs. thioactamide (TAA) group.			

Table I. Effects of treatment with ARBs on parameters of thioacetamide-induced testicular toxicity.



Fig 3. Sections of rat testis and cauda epididymis stained for collagen by Masson trichrome. (A) Normal Control group: The testis shows normal amount of collagen fibers separating seminiferous tubules (white arrow). The cauda shows fine collagen fibers along the tubular wall (black stars). (B)TAA group: The testis shows increases in collagen in the widely-spaced interstitial tissue (white arrows) and around the thickened congested blood vessels (BV). The cauda shows thickened collagen (black stars) around the tubules. (C) TAA+Candesartan group: The testis shows normal distribution of collagen (white arrows) between seminiferous tubules (ST) and the cauda shows normal distribution of collagen along and in-between tubular walls (black stars). (D) TAA+Losartan group: The testis shows moderate improvement with nearly normal distribution of collagen (white arrows) between seminiferous tubules (ST) and the cauda shows also moderate improvement normal distribution of collagen along tubular walls (black stars).



Fig 4. Sections of rat testis and cauda epididymis stained by PAS: (A) Normal Control group showing PAS positive reaction in the thin basal lamina of seminiferous tubules (white arrows) and mild positive reaction in interstitial connective tissue (black arrows). (B) TAA group showing increase in the positive PAS reaction in the thickened basal lamina of seminiferous tubules (white arrow) and degenerated germ cells and interstitial connective tissue (black arrows). (C) TAA+Candesartan group showing restoration of normal PAS reaction in the basal lamina (white arrow) nearly similar to control. (D) TAA+Losartan group showing normal PAS positive reaction in the basal lamina with focal increase in reaction in the interstitial tissue (black arrows). The cauda epididymis showed increase in PAS positive reaction in the basal lamina of tubules (black arrows) and cellular debris in TAA group (black stars) which appeared to be ameliorated by both candesartan and losartan.

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Fig 6. Sections from rat testis immunohistochemically stained for PCNA. (A) Normal Control group: the testis shows high immunopositive expression for PCNA in all the spermatogenic intact germ cells of lining the seminiferous tubules (black arrows). Few interstitial cells showed positive reaction (black stars). The cauda showed that most nuclei of lining epithelial cells of cauda tubules showed positive expression for PCNA (black arrows). (B) TAA group: the testis showed a decrease in PCNA reaction in the seminiferous tubule germ cells (black arrows) as most germ cells are degenerated. An increase in PCNA expression was observed in Leydig cells (black stars). The cauda showed negative expression for PCNA in degenerated swollen cells (arrows) while other cells showed normal expression. (C) TAA+Candesartan group: the testis showed restoration of immunopositive reaction for PCNA in the intact proliferating spermatogenic germ cells of the seminiferous tubules (black arrows). (D) TAA+Losartan group: the testis showed immunopositive reaction for PCNA in the intact proliferating germ cells of seminiferous tubules (black arrows). (D) TAA+Losartan group: the testis showed also PCNA expression in the nuclei of lining cells similar to control (black arrows) and few interstitial cells (black stars). The cauda showed also PCNA expression in the nuclei of lining cells nearly similar to control (black arrows) and few interstitial cells (black stars). The cauda showed also PCNA expression in the nuclei of lining cells nearly similar to control (black arrows) and few interstitial cells (black stars). The cauda showed also PCNA expression in the nuclei of lining cells nearly similar to control (black arrows).

### DISCUSSION

Liver fibrosis could decrease male fertility (Foresta et al.). The increased markers of lipid peroxidation and the decreased natural antioxidants in the testicular tissue supported the postulation of a direct toxic effect of TAA on testicular tissue via stimulating oxidative mechanisms (Kang et al.). The effect of TAA may be directly on the spermatogenic cells or via damage of their supporting Sertoli cells (Lenzi et al., 2002; Cheng, 2014). In the present study, the decrease in Leydig cells population could be explained in view of reported testosterone changes in patients with liver fibrosis (Nitsche et al.). It was found that exposure to flutamide during intrauterine life resulted in a chronic apoptotic germ cell death in the adult rat testicular tissue correlated with an increase in the expression and activation in germ cells of caspases-3 and -6 which are two important components in the apoptotic machinery (Omezzine et al., 2003). In rats, lead acetate significantly increased level of testicular caspase-3 expression. PCNA immunohistochemistry showed three differences between the testes of control and hypothyroid rats indicating its usefulness as a marker to differentiate between the two regarding germ cell kinetics and spermatogenesis (Tousson et al., 2011).

PAS was used in the present study to demonstrate any changes in the tubule basement membrane and the result showed that it was thickened in TAA group which may have a role in impaired spermatogenesis and germ cell degeneration. Thickening of basement membrane and excessive deposition of extracellular matrix proteins are characteristics of fibrotic disorders of multiple organs including liver and kidney (Rosenbloom et al., 2010). In rats, PAS staining was used as a histomorphological method to detect disorders in seminiferous tubular histoarchitecture (Ogedengbe et al., 2016). In the present study, both candesartan and lorsartan modulated the TAA-induced testicular toxicity and all histological and immunohistochemical alteration in both testicular tissue and cauda epididymis tubules with non-significant differences in-between. Candesartan relieved the cisplatin-induced testicular damage by modulating the expression patterns of the testicular nephrin-podocin complex (Enatsu et al., 2015). In rats, losartan partially reversed the angiotensin II type 1 receptor dependent increase in interstitial fibrosis and impairment of spermatogenesis that occurred postvasectomy due to oxidative stress (Shiraishi et al., 2003). Losartan protected against testicular germ cell apoptosis caused by an experimental varicocele (Bolat et al., 2016). Limitations of the current study include lack of quantitative histological analysis and use of only two ARBs members.

In conclusion, treatment with ARBs (candesartan and losartan) significantly reversed thioacetamide-induced lowgrade testicular and cauda toxicity in rats. This could be potentially useful for early treatment of male patients with occupational toxicant-induced reproductive dysfunction especially if they are using ARBs for other comorbidities.

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**FADDLADDEEN, K. A.; MURAD, H. A. M. & ALI, S. S.**Mejora de los cambios en la histoarquitectura con bloqueadores de receptores de angiotensina en la toxicidad precoz testicular y de cauda en ratas. *Int. J. Morphol., 37*(2):515-521, 2019.

**RESUMEN:** La disfunción reproductiva es una complicación por muchas enfermedades y toxinas. Su diagnóstico y tratamiento tempranos son inmensamente importantes. Aquí se evaluaron los cambios morfológicos en la histoarquitectura en la toxicidad precoz testicular y cauda antes y después del tratamiento con bloqueadores de receptores de angiotensina. Se indujo daño testicular de bajo grado usando tioacetamida (TAA, 50 mg / kg / día) por vía intraperitoneal durante dos semanas en ratas. Las ratas se dividieron aleatoriamente en cuatro grupos (n = 8) tratados diariamente por vía oral durante tres semanas de la siguiente manera: control normal (agua destilada), TAA (control positivo), TAA + candesartan (0,2 mg / kg) y TAA + losartán (7,5 mg / kg). Se midieron la testosterona sérica, el malondialdehído testicular y el glutatión. Los cambios en la histoarquitectura de los testículos y la epidermis de la cauda se evaluaron mediante Hematoxilina y Eosina para determinar la estructura general, con tricrómicro de Masson para el colágeno, ácido periódico de Schiff para la membrana basal y la caspasa-3 y el antígeno nuclear de células proliferantes (PCNA) para análisis inmunohistoquímico. Las ratas TAA mostraron disminución de la testosterona sérica y glutatión testicular, aumentos en el malondialdehído testicular, cambios degenerativos y apoptosis en células germinales, engrosamiento de la lámina basal tubular y aumentos en la expresión de la caspasa 3, y disminución en la expresión de PCNA. Los ARB (candesartán y losartán) revirtieron significativamente estos cambios con diferencias no significativas en el medio. El tratamiento con BRA (candesartán y losartán) revirtió significativamente la toxicidad testicular y cauda inducida por TAA en ratas. Esto podría ser potencialmente útil para el tratamiento temprano de pacientes con disfunción reproductiva inducida por tóxicos ocupacionales, especialmente si están usando BRA para otras comorbilidades.

PALABRAS CLAVE: Caspasa-3; Masson; Antígeno nuclear de células en proliferación; Bloqueadores de receptores de angiotensina. FADDLADDEEN, K. A.; MURAD, H. A. M. & ALI, S. S. Improved histoarchitectural changes with angiotensin receptor blockers in early testicular and cauda toxicity in rats. Int. J. Morphol., 37(2):515-521, 2019.

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