RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

DOI: 10.4274/atfm.galenos.2020.60362
Journal of Ankara University Faculty of Medicine 2020;73(3):290-299

MEDICAL SCIENCES / DAHİLİ TIP BİLİMLERİ

The Effect of Doxycycline on Glomerulosclerosis in 5/6 Renal Ablation Nephropathy

Doksisiklinin 5/6 Renal Ablasyon Nefropatisi ile Gelişen Glomerulosklerozа Etkisi

Devrim Kahraman[1], Ali Çelik[2], Funda Sağlam[3], Osman Yılmaz[4], Efsun Kolatan[4], Zahide Çavdar[5], Sulen Sarıoğlu[6]

[1]TOBB University Faculty of Medicine, Department of Pathology, Ankara, Turkey
[2]Dokuz Eylül University Faculty of Medicine, Department of Nephrology, İzmir, Turkey
[3]Aydın State Hospital, Clinic of Nephrology, Aydın, Turkey
[4]Dokuz Eylül University Faculty of Medicine, Department of Experimental Animals, İzmir, Turkey
[5]Dokuz Eylül University Faculty of Medicine, Department of Biochemistry, İzmir, Turkey
[6]Dokuz Eylül University Faculty of Medicine, Department of Pathology, İzmir, Turkey

Abstract

Objectives: The beneficial effect of matrix metalloproteinases (MMP) inhibitors in renal diseases has been reported. Their effect in segmental sclerosis is unknown. The aim of this study is to investigate the effect of a MMP inhibitor, doxycycline, on glomeruloscleresis (GS) in renal ablation nephropathy and to evaluate the MMP-2, MMP-9, tissue inhibitors of matrix metalloproteinases (TIMP)-1, TIMP-2, and collagen type IV expressions.

Materials and Methods: Fourteen of the 32 female Wistar albinos were 5/6 nephrectomised. Doxycyline was given to half of each group (40 mg/kg/day total 28 days). After sacrification, the GS, MMP-2 and MMP-9 expressions, and TIMP-2 expressions were analyzed histopathologically. Pro and active MMP-2 and -9 were analyzed by gelatin zymography. TIMP-1 and TIMP-2 were measured with the enzyme-linked immunoassay.

Results: Doxycycline administration to the 5/6 nephrectomy group improved GS but did not inhibit glomerular MMP-9 or cortical pro- and active-MMP-2 and pro-MMP9 but increased TIMP-1 and TIMP-2 expression in all groups in cortical tissue. Type IV collagen was decreased in the groups where GS were increased. MMP-9 expression and GS were increased in all groups receiving doxycycline.

Conclusion: We have demonstrated improved GS in renal ablation model by doxycycline administration but also doxycycline has an unexpected adverse effect. The effect of doxycycline on the expression of MMP-2 and -9 cannot explain the improvement in GS but increased cortical TIPM-1 and -2 may be an important contributing factor for the inhibition of MMPs. Other types of MMPs and TIMPs may be important. Accumulation of other types of collagen may be prominent in GS of ablation nephropathy.

Key Words: Matrix Metalloproteinases, Tissue Inhibitors of Matrix Metalloproteinases, Doxycycline, Renal Ablation Nephropathy

Özet

Amaç: Matriks metalloproteinaz (MMP) inhibitörlerinin böbrek hastalıklarına faydalı etkisi daha önce yayınlanmıştır. Ancak segmental skleroza olan etkileri belirsizdir. Bu çalışmanın amacı bir MMP inhibitörü olan doksisiklinin renal ablasyon nefropatisi ile oluşturulmuş glomerülosklerozа (GS) etkisi ve MMP-2, MMP-9, matriks metalloproteinaz doku inhibitörleri (TIMP)-1, TIMP-2 ve kollajen tip IV ekspresyonlarını incelemektir.

Gereç ve Yöntem: Çalışmamızda 32 adet dişi Wistar albino sıçanın 14'üne 5/6 nefrektomi uygulanmış; bunların ve nefrektomi uygulanmayanların yarısına oral doksisiklin verilmiştir (40 mg/kg/gün toplam 28 gün). Sakrifikasyon sonrası histopatolojik olarak glomerüllerdeki sklerotik değişikliklerin yanı sıra, immünfloresans ile MMP-9 düzeyleri ve immünhistokimyasal olarak TIMP-2 ekspresyonu değerlendirilmiştir. Ayrıca enzim bağlı immünosorbent deneyi ile, TIMP-1 ve TIMP-2 düzeyleri; jelatin zimografiyle pro ve aktif MMP-2 ve MMP-9 ölçülmüştür.

Bulgular: Araştırma sonucunda 5/6 nefrektomi modelinde doksisiklinin glomerulosklerozu azaltıcı etkisi saptanmış ancak glomerular MMP-9 ya da kortikal pro ve aktif MMP-2 ve pro-MMP9 inhibisyonu görülmemiştir. Tüm gruplarda kortikal dokuda TIMP-1 ve TIMP-2 artışı saptanmıştır. Tip IV kollajen glomerulosklerozun arttığı gruplarda azalmıştır. MMP-9 ekspresyonu ve glomerülosklerozda doksisiklin alan tüm gruplarda azalmıştır.

Address for Correspondence/Yazışma Adresi: Lect. Devrim Kahraman MD,
TOBB University Faculty of Medicine, Department of Pathology, Ankara, Turkey
Phone: +90 312 292 98 38 E-mail: dr.devrimsonmez@gmail.com ORCID: orcid.org/0000-0001-9858-9252
Received/Geliş: 06.08.2020 **Accepted/Kabul:** 12.08.2020

Sonuç: Doksisiklin uygulanması ile renal ablasyon modelinde glomerülosklerozun azaldığını gösterdik, ancak doksisiklinin beklenmedik bir yan etkisi görüldü. Doksisiklinin MMP-2 ve MMP-9 ekspresyonu etkisi glomerulosklerozdaki düzelmeyi açıklayamaz, fakat kortikal TIMP-1 ve TIMP-2'deki yükselme MMP inhibisyonundaki önemli bir faktör olabilir. Diğer MMP ve TIMP tipleri de önemli olabilir. Diğer kollajen tiplerindeki birikim ablasyon nefropatisinde meydana gelen glomerulosklerozda belirgin olabilir.
Anahtar Kelimeler: Matriks Metalloproteinaz, Matriks Metalloproteinaz Doku İnhibitörleri, Doksisiklin, Reanal Ablasyon Nefropatisi

Introduction

Matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinasas (TIMP) are important in the maintenance of extracellular matrix structure. Scar formation due to many diseases is related to imbalance between synthesis and degradation of extracellular matrix (1). MMP and TIMP expression are also related to tumor behavior in many types of carcinomas (2,3). The healing or improvement of injury by MMP inhibitors in renal diseases has been reported. Ahuja (4) reported a young man with crescentic nephritis, who was started a tetracycline group antibiotic doxycyclin, a nonselective MMP inhibitor, for steroid induced acne. Cessation of the drug was followed by increased and application resulted in decreased proteinuria. Afterwards Naini et al. (5) reported reduction of proteinuria of diabetic patients following administration of low dose doxycycline. Both the man described by Ahuja (4) and the diabetic patients had proteinuria as much as at the pretreatment period following cessation of doxycycline. On the other hand, there is no data about the effect of MMP inhibitors or doxycycline in segmental sclerosis. The aim of this study is to investigate the effect of doxycycline on glomerulosclerosis in renal ablation nephropathy and to evaluate MMP-9, MMP-2, TIMP-1 and -2, and collagen type IV (Col IV) which might have been be affected.

Materials and Methods

Animals

A total of 28 male Wistar albino rats (Experimental Animal Department of Dokuz Eylül University), weighing 200±20 g, were used throughout the experiment. Half of the rats were anesthetized with ether; the left renal pedicle was carefully dissected through a midline abdominal incision. Subtotal nephrectomy was performed for 14 rats by right nephrectomy followed by partial infarction of approximately two-thirds of the left kidney by selective ligation of two to three of three to four extrarenal branches of the left renal artery (6). They were kept on a 12-h light dark cycle at 20CK with 45% humidity in cages under free access to standard rat feed and tap water. Half of the nephrectomized (N) (group 1) and non-nephrectomised (ND) (group 4) rats received 0.9% saline and the others received doxycycline (groups 2 and 3 respesctively). Finally, the four groups were control (group C), only doxycyclin receving (Group D), only 5/6 nephrectomized (5/6N group) and 5/6 nephrectomized with doxycyclin (5/6ND group).

Administration of Doxycycline

Onset of treatment was the first date of the experiment. Doxycycline (40 mg/kg) was administered daily (in group 2 and 3), by gavage for 28 days.

Samples

All the rats were sacrificed following ether anesthesia on the day 28. For histopathological and biochemical examination kidneys were removed by a midabdominal incision. The kidneys of the rats were dissected and 2/3 of cortical tissue were examined histopathologically and the rest was spared for zymography and enzyme-linked immunoassay (ELISA).

Pathological Examination

Histopathological examination: 1/3 of the kidneys were fixed in formalin and embedded in paraffin. Two-micron meter thick tissue sections were taken on slides and stained with Hematoxylene eosin, periodic acid-Schiff, Masson's trichrome and periodic acid methanamine silver (PAMS).

Quantification of Glomerular Sclerosis

Segmental sclerosis rate was designated using the sections stained with hematoxylin and eosin and PAMS by the light microscopy by mapping and comparison of the glomeruli by both stains. According to the method described by Wu et al. (7) each glomerulus was graded as either normal (0), mildly sclerotic (1+, lesion occupying less than 50% of glomerular tuft), severely sclerotic (2+, lesion occupying more than 50% of glomerular tuft) or globally sclerotic (3+, lesion occupying 100% of glomerular tuft).

Immunohistochemistry

Sections from formalin fixed tissues were taken on poly-L-lysin coated slides and they were incubated in xylol for 20 minutes, followed 96%, 90%, 80% and 70% alcohol series respectively for 30 seconds. Later on, they were washed with tab water and boiled in citrate buffer for 15 minutes. After application of tris solution for five minutes and hydrogen peroxide for 1-minute tris solution was applied again for 10 minutes. Five minutes of protein blockage was followed with primary TIMP-2 (1/50, Neomarker) and type IV collagen (prediluted, Neomarker) antibody application for 1 hour. Tris wash, biotin, tris wash and streptavidine peroxidase were applied

for 10 minutes each. Following diaminobenzidine application Mayer's hematoxylene stain was applied and after 70%, 80%, 90% and 96% alcohol series; xylol was applied for 20 minutes. Expression for each antibody was evaluated by light microscopy in the glomeruli (0: negative, 1: mild, 2: moderate, 3: severe) as described previously (8).

Immunoflourescence

Non-fixed cortical renal materials were frozen in -50 centigrade with CO_2 jet and frozen sections were taken on poly-L-lysine coated slides. The sections were fixed by acetone and washed in phosphate buffered saline solution (PBS) and primary antibodies against, -pro and active MMP9 (1/50; (2C3): sc-21733;); Santa Cruz Biotechnology; Oregon, USA) and TIMP1 (1/50; Anti-TIMP-1 Antibody (2A5); Santa Cruz Biotechnology; Oregon, USA) were applied for 20 minutes at room temperature. PBS washes were followed by application of Fluorescein isothiocyanate conjugated secondary antibody (1/50, Eugene,Oregon, USA). After washing in PBS, the sections were cover-slipped by glysergel and the sections were evaluated by immunoflourescence microscopy. The staining intensity of the glomeruli was scored semiquantatively (0: negative, 1: mild, 2: moderate, 3: severe) for each antibody (9). MMP-2: The expression of MMP2 was evaluated by both immunohistochemistry and frozen section with direct immunofluorescence (DIF) methods but glomerular expression could not be identified in any cases, while there was positive staining of the control tissue and mild expression at the tubulo-intestitium.

Tissue Preparation for Biochemical Analysis

Tissue samples (cortex) were washed two times with cold saline solution and homogenized using a glass Teflon homogenizer (B. Brawn, Germany) in buffer at a ratio of 1/10 Tris HCl pH: 7.0, containing 10 mM $CaCl_2$, 0.05% Brij 35). The homogenate was then centrifuged at 10,000 xg for 10 minutes. The supernatants were used for gelatinases (MMP-2, MMP-9, active and -pro forms), TIMP-1 and TIMP-2 analyses as described below. All preparation procedures were performed at +4 °C. All homogenates were stored at –80 °C prior to testing.

Gelatin Zymography

Both the pro- and the active froms of MMP-2 and MMP-9 were analyzed using gelatin zymography (10). To measure the activities of the MMP's present in the supernatants, gels containing %7.5 polyacrylamide, %0.1 type I gelatin, and %10 sodium dodecyl sulfate (SDS) were prepared. Equal volumes of homogenate and a non-reducing sample buffer (2x) were mixed and applied to the wells so that each well contained 50 Rg protein. Electrophoresis was performed for 4 hours, at +4 °C, under a constant voltage of 125V (30 mA/gel). After electrophoresis gels were washed two times with 2.5% Triton X-100 for 15 min to remove SDS, and the gel was subsequently incubated in buffer containing 50 mM Tris-HCl (pH=7.6), 150 mM NaCl, 10 mM $CaCl_2$, 0.5 mM $ZnCl_2$ and 0.02% Brij-35 for 16 h at 37 °C. The following day, gels were stained for 1 hour (h) with staining solution (0.5% Coomassie brillant blue, MMP-2 and MMP-9 standard (CC073; Chemicon, CA) 40% methanol and 10% acetic acid) and destained in the same solution without Comassie brillant blue. MMP marker (Chemicon), containing both pro and active form of MMP-2 and MMP-9, was used. A clear zone in the blue background indicated the presence of gelatinolytic activity. Computerized densitometry was used to evaluate relative enzymatic activity (UVP BioImaging Systems with a LabWorks 4.6 Image Acquisition Software). The results were given in arbitrary unit per Rg protein.

TIMP-2 and TIMP-1 ELISA Assay

TIMP-2 and TIMP-1 analysis were effectuated on the homogenates of the samples (from all experimental conditions) using an ELISA-based kit (Amerhsam), according to the manufacturer's instructions. Duplicate measurements were done for each sample. The absorbances were measured by an automated ELISA reader (Biotek Instrument Inc, USA, Synergy HT). All results were expressed as levels in mg protein.

Statistical Analysis

The data were expressed as means ± standard deviation. Statistical significance was analyzed using Mann-Whitney U test with Bonferroni. A p value <0.05 was considered significant.

Ethics

The experimental design was approved by the Ethics Committee of Dokuz Eylül University, Faculty of Medicine (no: 73, date: 25.08.2006).

Results

Glomerulosclerosis

Glomerulosclerosis (GS) was observed both at the 5/6N group and 5/6ND group. Unexpectedly GS was identified at some cases of D group (Table 1). There was significant difference between four groups of animals for GS scores (Kruskal-Wallis test; p=0,000). GS scores were highest for 5/6N group (mean = 2.14±0.38) and doxycycline administration reduced GS in 5/6ND group (mean = 0.63±0.52) significantly (p=0.001) (Figure 1, 2).

Renal Scarring

Collagen Type IV

The mean value for scores for Col IV was highest for group C (mean: 2.83±0.41) and lowest for 5/6N group (mean: 1.71±0.76) (Table 1). There was significant difference between four groups (Kruskal-Wallis test; p=0.033), but after Bonferroni correction there was not significant difference between any groups (p>0.008, Mann-Whitney U test) (Figure 3, 4).

Table 1: Segmental sclerosis rate (SSR). Expression for antibodies (type IV collagen, MMP9 and TIMP2)

	Glomerulosclerosis	Type IV collagen	MMP-9	TIMP-2
5/6N	2.14±0.38	1.71±0.76	1.29±0.49	2.86±0.38
5/6ND	0.63±0.52	1.75±0.71	2.13±0.99	1.75±0.88
D	0.29±0.49	2.29±0.49	2.29±0.49	0.29±0.49
C	0	2.83±0.41	0	0.5±0.55
Total	0.79±0.92	2.11±0.74	1.5±1.07	1.39±1.2

MMP: Matrix metalloproteinases, TIMP: Tissue inhibitors of matrix metalloproteinasas, N: Nephrectomized, ND: Non-nephrectomised, D: Doxycyclin, C: Control

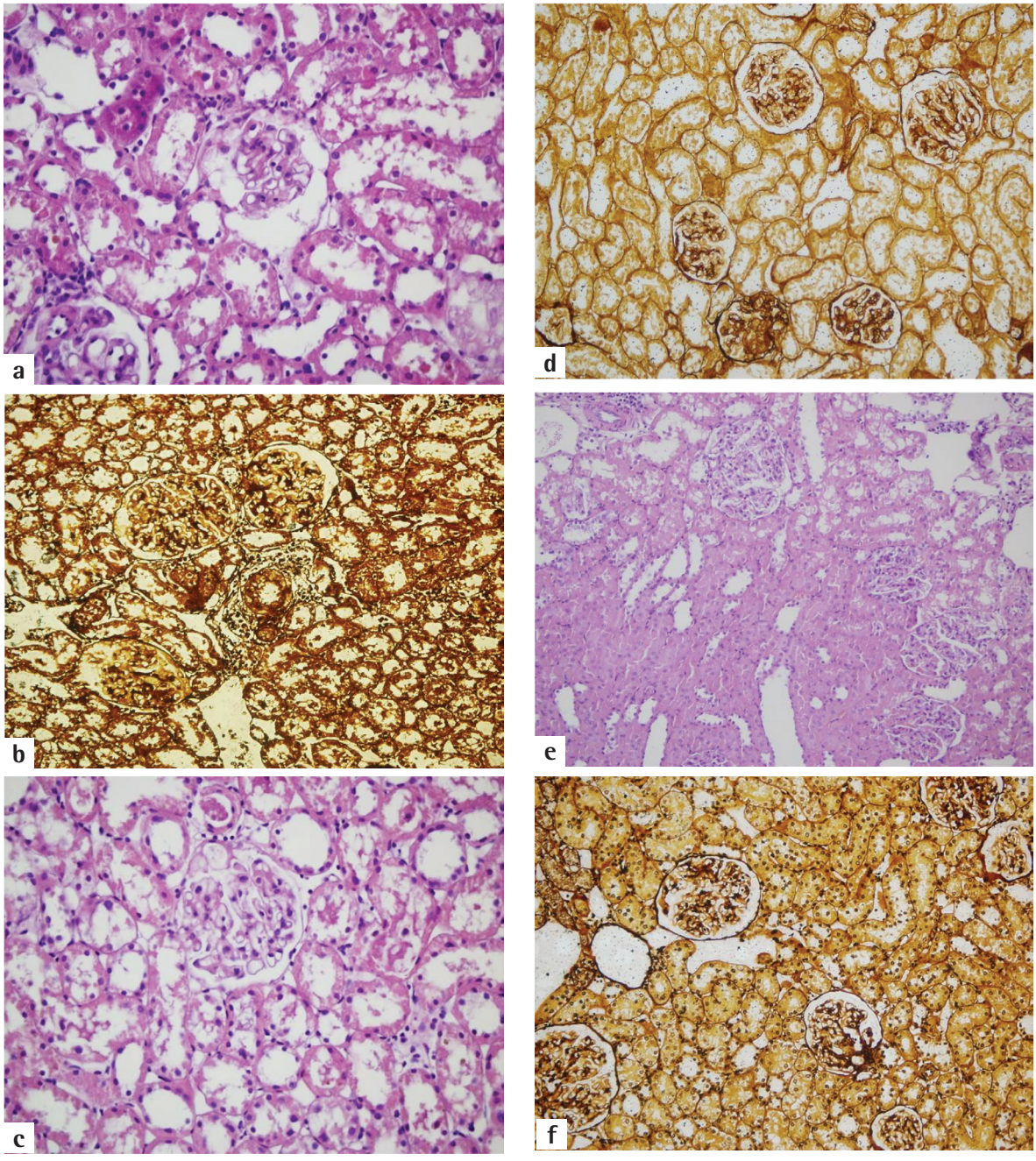

Figure 1: Glomerulosclerotic lesions in three groups: **a, b:** 5/6 neprectomized, **c, d**: 5/6 nephrectomized + doxycycline, **e, f:** doxycycline only, (a, c, e: H&Ex40, b, d, f: PAMSx20)
H&E: Hematoxylin and eosin, PAMS: Periodic acid methanamine silver

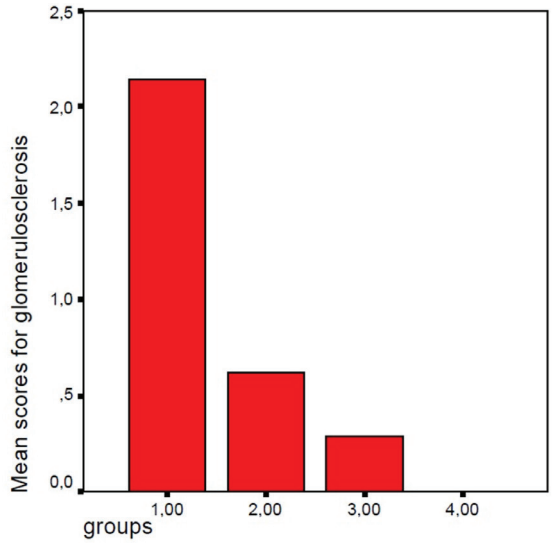

Figure 2: Mean glomerulosclerosis scores (groups 1: 5/6 neprectomized, 2: 5/6 nephrectomized + doxycycline, 3: doxycycline only, 4: control)

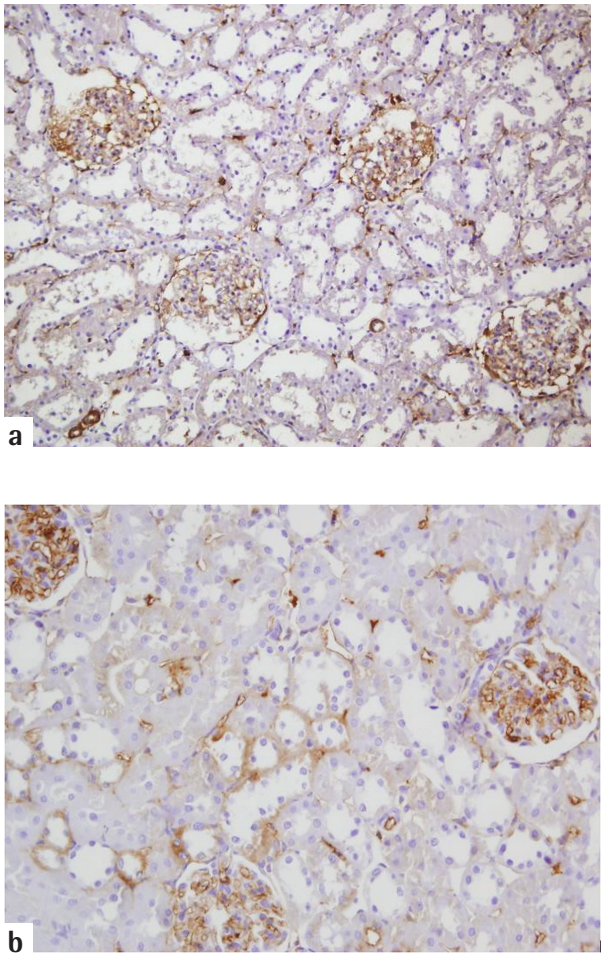

Figure 3: Collagen type IV expression in **a:** 5/6 neprectomized, (IHC anti collagen type IV, x20) **b:** control group (IHC anti collagen type IV, x40)

IHC: Immunohistochemistry

MMP-9

There was significant difference between four groups (Kruskal-Wallis test; p=0.000). The highest MMP-9 scores were identified for cases which received only D (mean: 2.29±0.49) followed by the 5/6ND group (mean: 2.13±0.99). All cases of the control group were negative for MMP-9 expression. There was only significant difference with control group and the other three groups (p=0.001, Mann-Whitney U test) (Figures 5, 6).

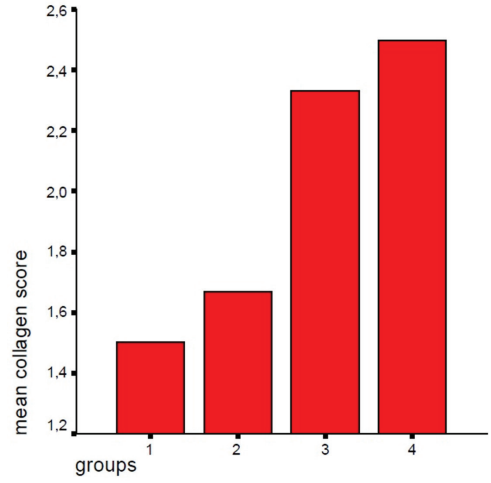

Figure 4: Mean collagen type IV expression scores

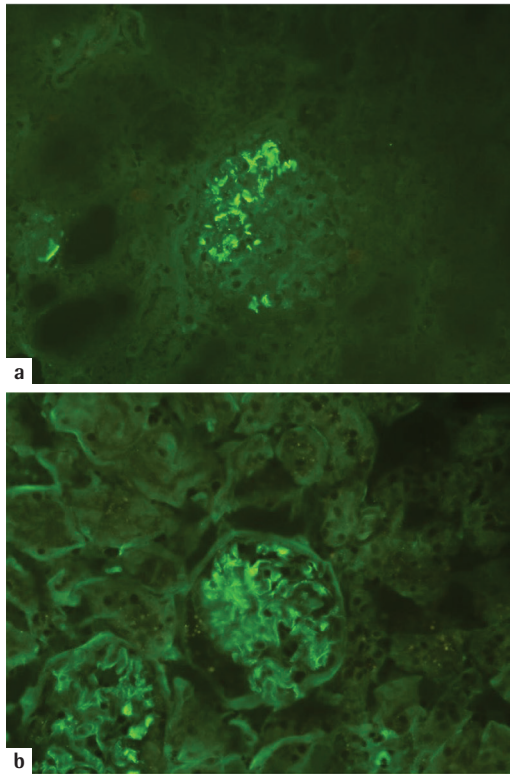

Figure 5: MMP-9 expression in four groups: **a:** 5/6 neprectomized, **b:** 5/6 nephrectomized + doxycycline, c: doxycycline only and d: control group (DIF, anti MMP-9, x40)

DIF: Direct immunofluorescence, MMP: Matrix metalloproteinases

TIMP-2

There was significant difference between four groups (Kruskal-Wallis test; p=0.000). The highest scores were identified for 5/6N group (mean = 2.86±0.38) and there was significant difference with 5/6ND, D and C groups (Mann-Whitney U test; p=0.007, p=0.001 and p=0.001, respectively) (Figures 7, 8).

TIMP-1 and TIMP-2 ELISA Assay

Cortical TIMP-1 and TIMP-2 activities were analyzed by ELISA. There was significant difference between groups (Kruskal-Wallis test; p=0.001 and 0.002 respectively) (Table 2). TIMP-1 and TIMP-2 were increased in 5/6 N, 5/6ND and D groups compared with the C groups (Mann-Whitney U test, p=0.003, 0.002 and 0.003 and, p=0.003, 0.002 and 0.003 respectively). There was not significant difference between 5/6N, 5/6ND and D groups (p>0.05) (Figures 9, 10).

Gelatin Zymography

Both the pro- and the active forms of MMP-2 and MMP-9 were analyzed using gelatin zymography (Figure 11) same as the literature (10). Pro MMP-9 was positive, but active MMP-9 was

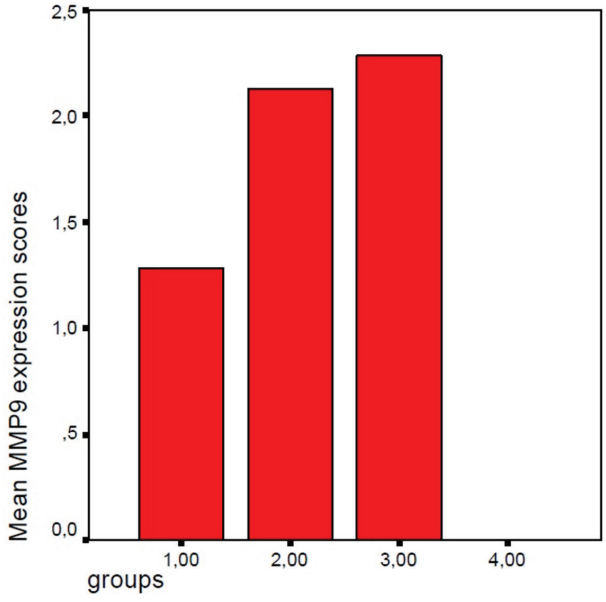

Figure 6: Mean MMP-9 expression scores

MMP: Matrix metalloproteinases

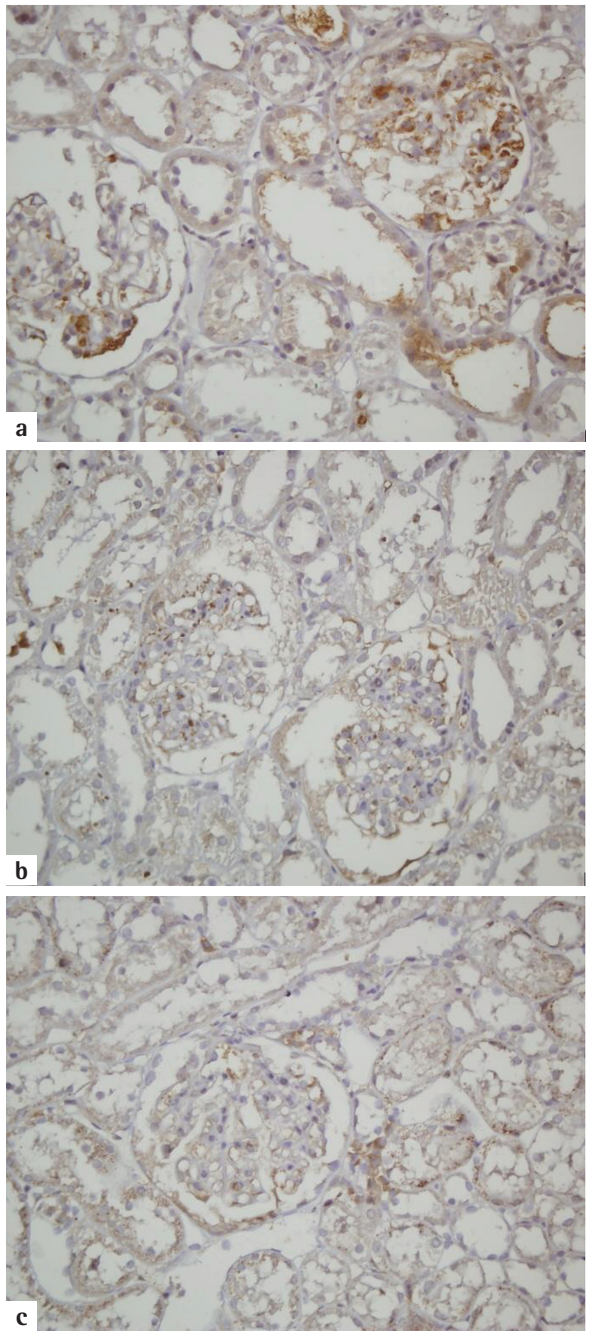

Figure 7: TIMP-2 expression: **a:** 5/6 neprectomized, **b:** 5/6 nephrectomized + doxycycline, **c:** doxycycline only group (IHC, anti TIMP-2, x40)

IHC: Immunohistochemistry, TIMP: Tissue inhibitors of matrix metalloproteinasas

Table 2: Expression for antibodies (TIMP1, bTIMP2, Pro MMP-2, Active-MMP, Pro MMP-9)

	TIMP1	bTIMP2	Pro MMP-2	Active-MMP-2	Pro MMP-9
5/6N	75.66±14.99	1,083.21±129.82	44,837.37±19,523.81	27,983.02±25,607.06	1,273,419.07±335,651.37
5/6ND	167.30±243.47	1,201.7±137	94,796.95±46,939.77	54,216.6±22,105.32	1,567,953.69±285,709.24
D	78.81±7.7	1,164.74±182.04	52,152.76±15,107.95	23,687.35±10,954.45	898,070.5514±130,705.99
C	45.70±2.82	662.40±73.23	94,249.79±21,957.19	18,191.06±10,326.74	1,441,712.67±568,313.80
Total	96.21±132.99	1,047.27±246.70	71,528.75822143±36,784.81709382	32,306.14±23,009.52	1,299,797.60±421,437.16

MMP: Matrix metalloproteinases, TIMP: Tissue inhibitors of matrix metalloproteinasas, N: Nephrectomized, ND: Non-nephrectomised, D: Doxycyclin, C: Control

negative in all groups. Only for the D group the active form was significantly decreased compared with 5/6N, 5/6ND (p=0.006, p=0.001) (Table 2, Figure 12). Pro-MMP-2 was also significantly different for all groups (Kruskal-Wallis test; p=0.001). The highest values were identified for 5/6ND and C groups. Pro-MMP2 was decreased for 5/6N and D groups compared with 5/6ND and C groups (p<0.008) (Figure 13). Active MMP-2 was significantly different for all groups (Kruskal-Wallis Test; p=0,001), but after Bonferroni correction there was not significant difference between any groups (p>0.008, Mann-Whitney U test) (Figure 14).

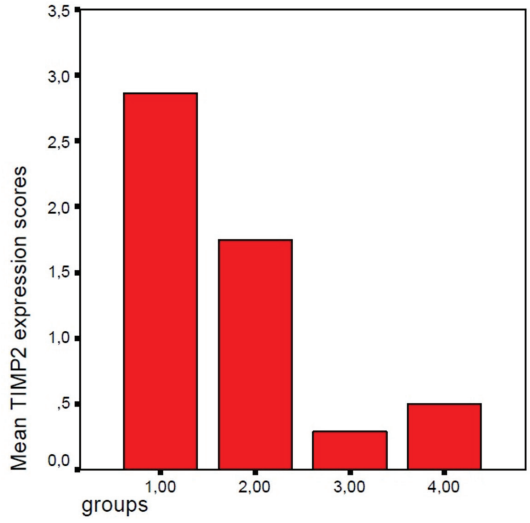

Figure 8: Mean TIMP-2 expression scores

TIMP: Tissue inhibitors of matrix metalloproteinasas

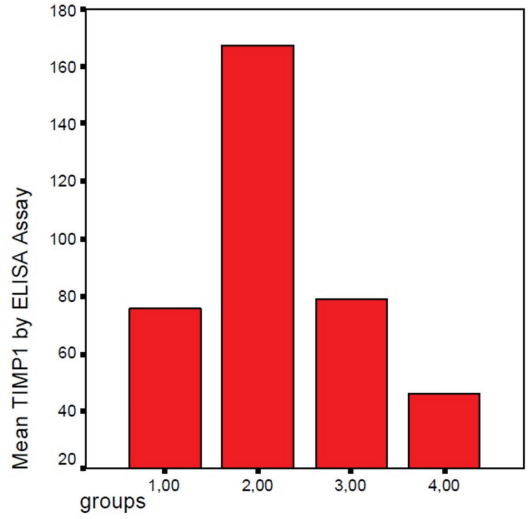

Figure 9: Mean TIMP-1 by ELISA assay

TIMP: Tissue inhibitors of matrix metalloproteinasas, ELISA: Enzyme-linked immunoassay

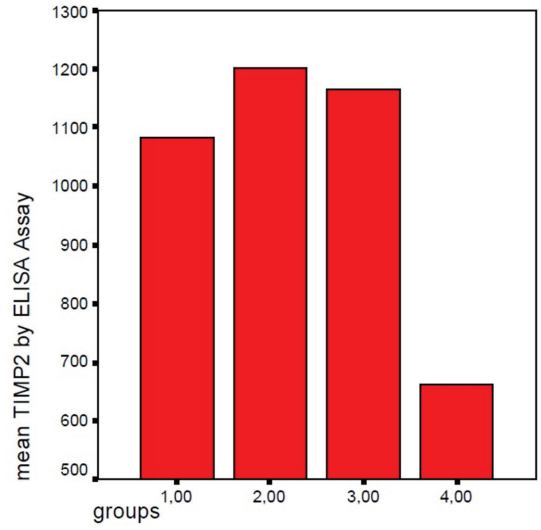

Figure 10: Mean TIMP-2 by ELISA assay

TIMP: Tissue inhibitors of matrix metalloproteinasas, ELISA: Enzyme-linked immunoassay

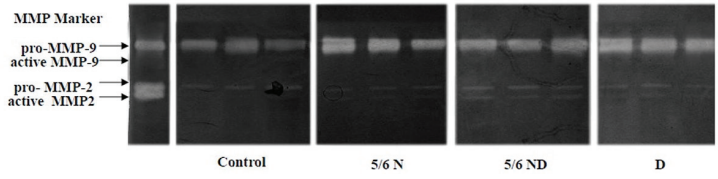

Figure 11: Representative gelatin zymogram patterns of control, 5/6N, 5/6ND and D groups. Positions of pro MMP-9 (92 kDa), active MMP-9 (82 kDa) and pro-MMP-2 (72 kDa), active MMP-2 (62 kDa) are indicated in MMP marker line

N: Nephrectomized, ND: Non-nephrectomised, D: Doxycyclin, MMP: Matrix metalloproteinases

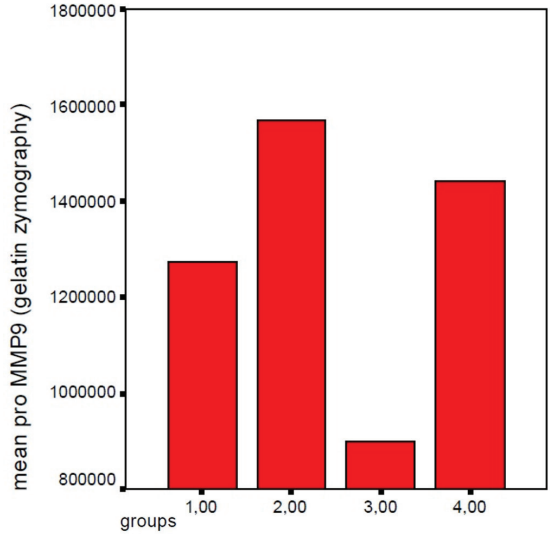

Figure 12: Mean pro MMP-9 by gelatin zymography

MMP: Matrix metalloproteinases

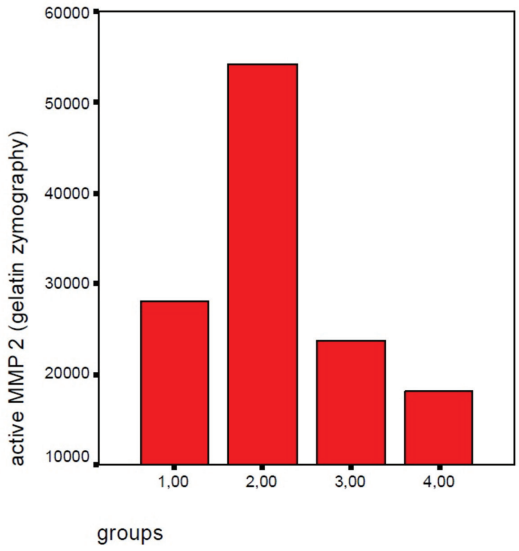

Figure 13: Mean pro MMP-2 by gelatin zymography
MMP: Matrix metalloproteinases

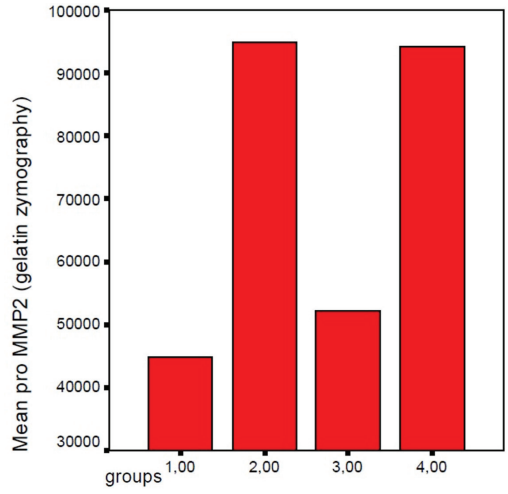

Figure 14: Mean active MMP-2 by gelatin zymography
MMP: Matrix metalloproteinases

Discussion

The changes of MMPs and TIMPs have been documented during glomerulonephritis both in the plasma and renal tissue (11). Ahuja et al. (11) evaluated the mRNA levels of HIV associated nephropathy in renal biopsies and identified increased expression of MMP-9 in all patients. Liu et al. (12) reported the MMP-9 expression in an experimental model of puromysin aminonucleoside and identified increase of pro but decreased active MMP-9 and they could not demonstrate glomerular MMP-2 expression. Johnson et al. (13) evaluated the RNA expression in 5/6 nephrectomy cases and identified increased collagen type I, III and IV as well as MMP-2 and TIMP-1, late TIMP-2 expression, but not MMP-9. Bauvois et al. (14) evaluated the plasma concentrations of patients with glomerulonephritis and in a series of 108 patients and identified increased MMP-2 and TIMP-1 and unchanged MMP-9 in minimal change/focal segmental sclerosis patients. Ahmed et al. (15) reported increased glomerular MMP-1 expression in 5/6N experimental model. In this 5/6 nephrectomy experimental model we could not demonstrate glomerular MMP-2 expression like Liu et al. (12) but we have identified significantly increased MMP-9 expression in 5/6N group by direct immunofluorescein, while all the cases from the control group were negative. The active form of MMP-9 was negative in all groups and the pro-MMP9 was decreased in 5/6N compared with the C group. ProMMP-2 was significantly decreased in 5/6N compared to C group and the active form was also slightly increased suggesting both activation and consumption of MMP2 in 5/6N group. These controversial but complementary histopathological and biochemical results may be related to the inclusion of all structures in biochemical analysis while the scores by DIF reflected only the glomeruli. Considering the DIF and biochemical results and the literature about the focal segmental sclerotic lesions (11), it seems increased glomerular expression of MMP9 is a feature of 5/6N experimental model. TIMP-1 and TIMP-2 were also increased for cases with 5/6N compared to the C group like MMP-9. Considering immunohistopathological and biochemical results in this series, in 5/6N experimental model MMP-9 and TIMP-1 and TIMP-2 expressions are increased, while glomerulosclerosis is increased. We evaluated the expression of Col IV which is known to be a target of MMP-2 and MMP-9 and observed decreased glomerular expression in 5/6N compared with C by immunohistochemistry (12). There are controversial results about the type of collagen deposited during glomerulosclerosis. Cai et al. (16) reported increased type IV and VI collagens in cases with focal segmental sclerosis but Olgemöller et al. (17) identified increased glomerular type III collagen in diabetic nephropathy. Johnson et al. (13) reported increased collagen I, III and IV RNA expression in 5/6N experimental model. It seems other collagen types than type IV might be expressed contributing to sclerotic lesions as we have identified decreased Col IV in 5/6N group. The increased MMP-9 and TIMP-2 expressions and decreased col IV are in concordance with this series. Also, our gelatin zymogram results show that decreased Col IV expression due to increased proMMP-9 activity is related to low TIMP-1 protein levels in 5/6N group. We have also evaluated the effect of a MMP inhibitor doxycycline in this series. Improved glomerulosclerosis in 5/6ND group compared to 5/6N group was identified, but this could not be explained by the suppression of MMP-9 or MMP-2 as both were increased. Increased TIMP-2 and especially TIMP-1 was identified in 5/6ND group compared with 5/6N group by ELISA assay. These findings suggest increased expression of MMP inhibitors might have contributed to the decreased GS even if MMPs are increased by blocking them. We don't know

exactly the effect of doxycycline on TIMPs, but these findings suggest that doxycycline may also induce their expression. The semiquantitative scores for Col IV were not significantly different for 5/6N and 5/6ND groups but the glomerulosclerosis scores were significantly higher for 5/6N group. These findings suggest that doxycycline administration have beneficial effects on GS in 5/6N model and MMPs might be important during this process. We identified increased glomerulosclerosis in D group compared with C, suggesting an unexpected adverse effect of doxycycline on glomeruli. Previously decreased Col IV with doxycycline administration was reported (18). We found also increased TIMP-1 and TIMP-2 protein level but decreased proMMP-9 and proMMP-2 activity level in D group. MMP are known to be highly regulated at transcriptional, translational and activity levels which may account for the differences between the levels of MMP-9 protein expression and proenzyme activity in D group (19). Inhibition of MMP-9 with doxycycline most likely occurs through either direct blockade of enzyme activity or prevention of pro-MMP activation. Previous studies have reported that doxycycline causes conformational changes and loss of enzymatic activity of MMPs by binding to the active zinc site and secondarily to the inactivated calcium ion site (20,21).

One shortcoming of this series is that, we did not evaluate the expression pattern of MMP-14 which is a component of slit diaphragm related to minimal lesion disease and focal segmental sclerosis probably important in effacement of pedicelles, also, an important in extracellular matrix degradation and a MMP-2 activator (19).

Conclusion

The results of this series suggest that 5/6 nephrectomy renal ablation model is associated with increased glomerular MMP-9 and TIMP-2 expression and decrease in Col IV. Doxycycline an inhibitor of MMPs improves GS, but it has adverse effects on glomeruli so the effect of other MMP inhibitors might be evaluated in similar experimental models (22,23). The effect of doxycycline expression on MMP-2 and -9 as well as the TIMP-1 and -2 presented in this series cannot explain the improvement in GS suggesting the role of other MMPs and TIMPs which require further research.

Ethics

Ethics Committee Approval: The experimental design was approved by the Ethics Committee of Dokuz Eylül University, Faculty of Medicine (no: 73, date: 25.08.2006).

Informed Consent: Experimental study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: O.Y., E.K., D.K., Concept: S.S., A.Ç., G.O., Design: S.S., Data Collection or Processing: D.K., Z.Ç., Analysis or Interpretation: D.K., Z.Ç., Literature Search: F.S., Writing: D.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 1991;5:2145-2154.
2. Sarioglu S, Ozer E, Kirimca F, et al. Matrix metalloproteinases-2 expression in laryngeal preneoplastic and neoplastic lesions. Pathol Res Pract. 2001;197:483-486.
3. Sis B, Sagol O, Küpelioglu A, et al. Prognostic significance of matrix metalloproteinases-2, cathepsin D, and tenascin-C expression in colorectal carcinoma. Pathol Res Pract. 2004;200:379-387.
4. Ahuja TS. Doxycycline decreases proteinuria in glomerulonephritis. Am J Kidney Dis. 2003;42:376-380.
5. Naini AE, Harandi AA, Moghtaderi J, et al. Doxycycline: a pilot study to reduce diabetic proteinuria. Am J Nephrol. 2007;27:269-273.
6. Güray M, Sarioglu S, Türkmen M, et al. Cyclosporine A toxicity in association with reduced renal mass. Transplant Proc. 2003;35:3128-3133.
7. Wu LL, Cox A, Roe CJ, et al. Transforming growth factor beta 1 and renal injury following subtotal nephrectomy in the rat: role of the renin-angiotensin system. Kidney Int. 1997;51:1553-1567.
8. Tatekawa Y, Kemmotsu H, Joe K, et al. Matrix Metalloproteinase-9 Expression in Congenital Diaphragmatic Hernia During Mechanical Ventilation. 2005;35:524–529.
9. Sanders J, Goor H, Hanemaaijer R Renal expression of matrix metalloproteinases in human ANCA-associated glomerulonephritis., Nephrol Dial Transplant. 2004:1:1412-1419.
10. Kleiner D, Stetler-Stevenson WG. Quantitative Zymography: Detection of Picogram Quantities of Gelatinases. Anal Biochem. 1994;1;218:325-329.
11. Ahuja TS, Gopalani A, Davies P, et al. Matrix metalloproteinase-9 expression in renal biopsies of patients with HIV-associated nephropathy. Nephron Clin Pract. 2003;95:c100-c104.
12. Liu S, Li Y, Zhao H, et al. Increase in extracellular cross-linking by tissue transglutaminase and reduction in expression of MMP-9 contribute differentially to focal segmental glomerulosclerosis in rats. Mol Cell Biochem. 2006;284::9-17.
13. Johnson TS, Haylor JL, Thomas GL, et al. Matrix metalloproteinases and their inhibitions in experimental renal scarring. Exp Nephrol. 2002;10:182-195.
14. Bauvois B, Mothu N, Nguyen J, et al. Specific changes in plasma concentrations of matrix metalloproteinase-2 and -9, TIMP-1 and TGF-beta1 in patients with distinct types of primary glomerulonephritis. Nephrol Dial Transplant. 2007;22:1115-1122.
15. Ahmed AK, Haylor JL, El Nahas AM, et al. Localization of matrix metalloproteinases and their inhibitors in experimental progressive kidney scarring. Kidney Int. 2007;71:755-763.
16. Cai YI, Sich M, Beziau A, et al. Collagen distribution in focal and segmental glomerulosclerosis: an immunofluorescence and ultrastructural immunogold study. J Pathol. 1996;179:188-196.
17. Olgemöller B, Schleicher E. Alterations of glomerular matrix proteins in the pathogenesis of diabetic nephropathy. Clin Investig. 1993;71(5 Suppl):S13-S19.

18. Floege J, Johnson RJ, Gordon K, et al. Increased synthesis of extracellular matrix mesengial proliferative nephritis. Kidney Int 1991;40:477-488.
19. Munkert A, Helmchen U, Kemper MJ, et al. Characterization of the transcriptional regulation of the human MT1-MMP gene and association of risk reduction for focal-segmental glomerulosclerosis with two functional promoter SNPs. Nephrol Dial Transplant. 2009;24:735-742.
20. Björklund M, Koivunen E. 2005 Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta. 25;1755:37-69.
21. Hanemaaijer R, Visser H, Koolwijk P, et al. Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. Adv Dent Res. 1998;12:114-118.
22. Golub LM, McNamara TF, Ryan ME, et al. Adjunctive treatment with subantimicrobial doses of doxycycline: effects on gingival fluid collagenase activity and attachment loss in adult periodontitis. J Clin Periodontol. 2000;28:146-156.
23. Lutz J, Yao Y, Song E, et al. Inhibition of matrix metalloproteinases during chronic allograft nephropathy in rats.Transplantation. 2005; 27;79:655-661.