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The effectiveness of chitosan-mediated silencing of PDGF-B and PDGFR-β in the mesangial proliferative glomerulonephritis therapy

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ABSTRACT

Platelet-derived growth factor-B (PDGF-B) is a growth factor that plays an important role in the progression of mesangial proliferative glomerulonephritis (MsPGN). PDGF-B may contribute to mesangioproliferative changes and is overexpressed in MsPGN. Recently, small interfering RNAs (siRNAs) have been widely used for gene silencing effects in experimental models of renal diseases. Nanoparticle-based therapeutics are preferred for reasons such as increasing therapeutic efficacy and reducing toxic effects caused by high doses. The distribution of nanoparticles to the kidney is a significant advantage in siRNA delivery. The aim of this study was to investigate the efficacy of chitosan/siRNA nanoplexes in silencing of PDGF-B and PDGFR-β genes in kidney and to decrease mesangial cell proliferation and matrix accumulation in MsPGN model induced by anti-Thy-1.1 antibody. The therapeutic effects of chitosan/siPDGF-B + siPDGFR-β nanoplexes in glomerulonephritic rats were studied by molecular, biochemical, and histopathologic evaluations. Chitosan/siPDGF-B + siPDGFR-β nanoplexes markedly reduced PDGF-B and PDGFR-β mRNA and protein expressions in experimental MsPGN model. Histopathologic examination results showed that the silencing of PDGF-B and its receptor PDGFR-β led to reduction in mesangial cell proliferation and matrix accumulation. The use of chitosan/siPDGF-B + siPDGFR-β nanoplexes for silencing the PDGF-B pathway in MsPGN can be considered as a new effective therapeutic strategy.

1. Introduction

The prevalence of end-stage kidney disease is increasing day by day especially in the elderly population due to the risk factors such as hypertension and diabetes. The mesangioproliferative glomerulonephritis (MsPGN), a kidney disease has a high incidence (Stevens et al., 2010; Sotiriou and Piccart, 2007). It is characterized by the proliferation of mesangial cells and accumulation of extracellular matrix (Guo et al., 2017). The best-known growth factors involved in mesangial cell proliferation are members of the PDGF family (PDGF-A, -B, -C, and -D). PDGFs are mitogenic and important survival factors for mesangial cells that are activated by glomerulonephritis-induced interactive process and inflammatory events (Cove-Smith and Hendry, 2008). The increased in the expressions of PDGF-A, -B and PDGF receptors (PDGFRs) in glomerulonephritis have been demonstrated in many human and experimental models.

It is known that PDGF-B mediates glomerular mesangial cell proliferation through PDGFR-β. Many studies suggest that PDGF inhibition has a renoprotective effect in renal diseases (Boor et al., 2014; Gesualdo et al., 1994; Venkatesan et al., 2008). However, the specific effects of PDGF inhibition on both glomerular diseases and mesangial cell proliferation and activation are still poorly understood (Boor et al., 2014).

RNA interference (RNAi) is a specific and effective sequence-specific post-transcriptional gene silencing process, therefore it has been investigated as a new strategy in the treatment of kidney diseases in recent years (Kreidberg, 2010). RNAi studies with TGF-β have shown that this growth factor plays key role in fibrosis. Hwang et al. (2006) suppressed transforming growth factor beta (TGF-β) expression in fibrosis-induced kidney using shTGF-β1 vector. By this way, tubulointerstitial fibrosis in the kidney was prevented. Takabatake et al. (2007) showed that proteinuria and mesangial matrix deposition in glomeruli was decreased upon TGF-β1 siRNA treatment in an anti-Thy-1 glomerulonephritis model. PDGF can induce the expression of TGF-β and its

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receptor (Mima et al., 2011). The renoprotective effects of receptor tyrosine kinase inhibitors, antagonists, and aptamers against over-expressed PDGF-B and PDGFR-β in experimental models and human glomerulonephritis were investigated with promising results (Boor et al., 2014; Floege et al., 2008; Iyoda et al., 2009, 2013). However, to date, RNAi-mediated gene silencing of PDGF-B and PDGFR-β has not been studied in glomerulonephritis.

The most important barrier to siRNA applications in clinical trials is the lack of stability of the siRNA because of their they have poor pharmacokinetic properties (Xie et al., 2006). The renal clearance of naked siRNA is very rapid. Therefore, the development of appropriate gene carrier systems for siRNAs are required for delivery on target tissues and efficient gene silencing. Complexing negatively charged nucleic acid molecules with positively charged carrier systems facilitates cell entry. In particular, it has been reported that cationic non-viral carrier systems interact with glomerular basement membrane (Williams et al., 2016; Stokman et al., 2010; Scindia et al., 2010). Chitosan is a biodegredable, biocompatible, non-toxic and non-immunogenic cationic polymer. In several studies, it has been reported that low molecular weight chitosan can be used for delivery of therapeutic molecules into the kidneys (Gao et al., 2009, 2014; Yuan et al., 2007, 2009; Şalva et al., 2016, 2017). *In vitro* studies by Şalva et al. (2017) have shown that chitosan/siPDGFB + siPDGFR-β nanoplexes suppressed cell proliferation and migration by inhibiting PDGF-B expression in mesangial cells. *In vivo* biodistribution study by Şalva et al. (2016) have demonstrated siRNA accumulation in kidney tissue at 4 h after intravenous injection of chitosan/FITC-siPDGF-B nanoplexes. In the present study, chitosan nanoplexes containing siPDGF-B and siPDGFR-β using simple complexation method were developed. The therapeutic effects of chitosan nanoplexes in rat MsPGN model were investigated by molecular, biochemical and histopathologic studies.

2. Materials and methods

2.1. Materials

siRNAs targeting PDGF-B and PDGFR-β were obtained from Qiagen (USA). Chitosan (75 kDa; 75%–85% deacetylation) was purchased from Sigma (St. Louis, MO, U.S.A.). All the substances used in the study were of molecular biology grade. The anti-rat CD90 (Thy 1.1) monoclonal antibody that was purified by inducing glomerulonephritis (GN) was purchased from Cedarlane (Canada). Anti-PDGF-B and anti-PDGFR-β antibodies from Abcam, TUNEL assay kit from Roche and real-time PCR reagents and primers from Invitrogen were purchased.

2.2. The preparation and control of chitosan nanoplexes

Chitosan/siRNA nanoplexes were prepared by ionic complexation between anionic siRNA and cationic chitosan. 2.5 mg/ml solution of chitosan was prepared in acetic acid 1% (*v*/v) and filtered through 0,22 μm. siRNA was diluted in DNase/RNase free water. The nanoplexes were prepared by adding of chitosan solution to siRNA solution and vortexed. Then nanoplexes were incubated at room temperature for 1 h. The nanoplex formation were observed by agarose gel electrophoresis (2.5%).The particle size and zeta potential of nanoplexes were determined in PBS buffer (pH 7.4) by a zeta sizer (Malvern Instruments, NanoZS ZEN 3600).

2.3. Experimental glomerulonephritis model

Thy1.1 glomerulonephritis model (Thy1.1 GN) was induced by a single intravenous injection of monoclonal anti-Thy1.1 antibody OX-7 (1 mg/kg, Cedarlane) in the Sprague–Dawley male rats (Kagami et al., 2006). Rats (n = 6; 180–200 g initial body weights) were kept in the animal laboratory under controlled-environment conditions and received food and water *ad libitum*. All animal experiments were performed in accordance with the Animal Ethics Committee of Inonu University (2012/A-76, Inonu DHEK).

Rats were randomly divided into five groups:

(1) control group (n = 5) in which the rats were injected with PBS (0.5 ml/100 g body wt);
(2) Thy-1.1 GN model group (n = 5) in which the rats were given anti-Thy1.1 (0.5 ml/100 g body wt) by a single intravenous injection;
(3) the chitosan/siPDGFB + siPDGFR-β nanoplexes + Thy-1 nephritis group;
(4) the free siPDGFB + siPDGFR-β + Thy-1 nephritis group, and
(5) the chitosan + Thy-1 nephritis group. After administration of the anti-Thy1.1 antibody, siPDGFB + siPDGFR-β nanoplexes, free siRNAs and chitosan solutions were intraperitonally given every 3 days until day 10.

2.4. Biochemical study

To investigate the effect of chitosan/siRNA nanoplexes on proteinuria and creatinine in urine and creatinine in serum, rats were placed to metabolic cages with free access to water but without food and 24 hours urine samples were collected on day 10. Urinary protein concentration was determined by Bradford method in 24 hour urine samples (Westerweel et al., 2012). Urinary and plasma creatinine levels were measured using an autoanalyzer. Creatinine clearance (CrCl) was calculated as the floowing formula (Urine creatinine levels/serum creatinine levels) × 24-h urine volume (ml)/(24 h × 60 min) (Gu et al., 2014).

2.5. Histopathologic studies

For histopathologic evaluation, kidneys were harvested on day 10 and tissues were collected in neutral-buffered formalin then processed and embedded in paraffin by standard histological methods. The kidneys were sectioned into 4–5 μm thickness and Hematoxylin&Eosin (H&E) and PAS (Periodic Acid-Schiff) stainings were performed to examine pathologic changes under the light microscope. The mesangial cell proliferation was evaluated on ten separate fields (400× magnification) and histological evaluations were averaged (Geng et al., 2016). Glomerular matrix expansion was evaluated in 30 glomeruli where the percentage of mesangial matrix occupying each glomerulus was rated on a 0–4 scale where 0 = 0, 1 = 25, 2 = 50, 3 = 75, 4 = 100% (Huang et al., 2006).

The streptavidin-biotin peroxidase immunohistochemical staining method was performed to demonstrate PDGF-B and PDGFR-β immunostaining in the tissue. The sections were treated with 3% hydrogen peroxide for 20 min to suppress endogenous peroxidase activity. The nonspecific background was blocked by serum-free protein block. The primary antibodies against PDGF-B (anti-PDGF BB antibody, ab16829, diluted 1:100) and PDGFR-β (anti-PDGF receptor beta antibody, ab32570, diluted 1:200) were added on each section (Abcam, Cambridge, UK) and incubated at room temperature for an overnight incubation followed by three washes in TBS. Sections were incubated with rabbit specific HRP/DAB detection kit (ab80437, Abcam) at 37 °C for 20 min. After TBS, the sections were treated with DAB for 5 min and Mayer's hematoxylin was used to counterstain the cells. Coverslips were mounted on glass slides using mounting media. PDGF-B and PDGFR-β immunostaining were evaluated microscopically by choosing the hot point three regions (×400 magnification). The degree of immunohistochemical staining in kidney tissues was semiquantitatively evaluated between the groups. Staining intensity; 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) (Klopfleisch, 2013).

2.6. TUNEL assay

DNA fragmentation (terminal deoxynucleotide transferase-mediated

dUTP-biotin nick-end labeling (TUNEL) staining) was determined using ApopTag kit on parafin-embedded sections according to the manufacturer's instructions (Millipore, USA). The counterstaining was performed by Mayer Hematoxylin. The number of apoptotic glomerular cells was expressed as the mean number per glomerular cross-section.

2.7. Quantitative real-time PCR analysis

The quantification of PDGF-B ve PDGFR-β expressions in tissue was performed using real-time PCR (Step One Plus Applied Biosystem, USA). Beta-actin was co-amplified as an internal control. The relative level of gene expression was obtained by calculating ratio of cycle numbers of the initial exponential amplification phase as determined by the sequence detection system for specific target genes (PDGF-B ve PDGFR-β) and beta-actin. Fold changes were calculated using the relative C method.

2.8. Statistical analysis

The results are expressed as the arithmetic mean ± standard deviation. Statistical analysis of differences between two groups was performed using parametric Student's *t*-test. *P* values < .05 were considered as statistically significant.

3. Results

In this study, we have studied the effects of the siRNA containing chitosan nanoplexes targeted to PDGF-B and its receptor PDGFR-β on anti-Thy1 glomerulonephritis by analyzing effects of nanoplexes on mesangial cell number and mesangial matrix increase, on PDGF-B and PDGFR-β expressions and on renal functions by histopathological, molecular and biochemical methods, respectively. Our results show that chitosan/siPDGF-B + siPDGFR-β nanoplexes have remedied adverse changes in histopathologic and renal functions in glomerulonephritis.

3.1. In vitro controls of chitosan nanoplexes

The chitosan and siRNA complex formation was confirmed by gel electrophoresis. The full complex formation between siRNAs and chitosan at 5/1, 10/1, 15/1 and 20/1 ratios are shown in Fig. 1 and 20/1 weight ratio was selected for *in vivo* studies. The zeta potential value of the chitosan/siPDGF-B + siPDGFR-β nanoplexes at 20/1 weight ratio

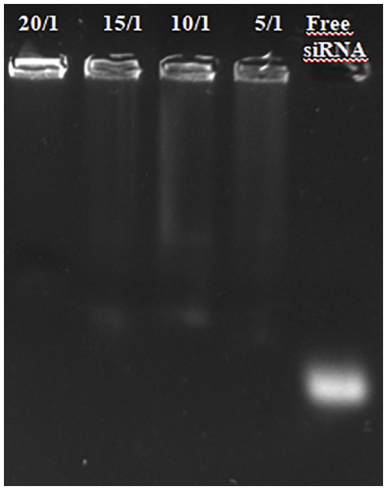

Fig. 1. Agarose gel electrophoresis of chitosan/siPDGF-B + siPDGFR-β nanoplexes. Free siRNA and nanoplexes in the different weight ratios (chitosan/siRNA; 5/1, 10/1, 15/1, 20/1) were given.

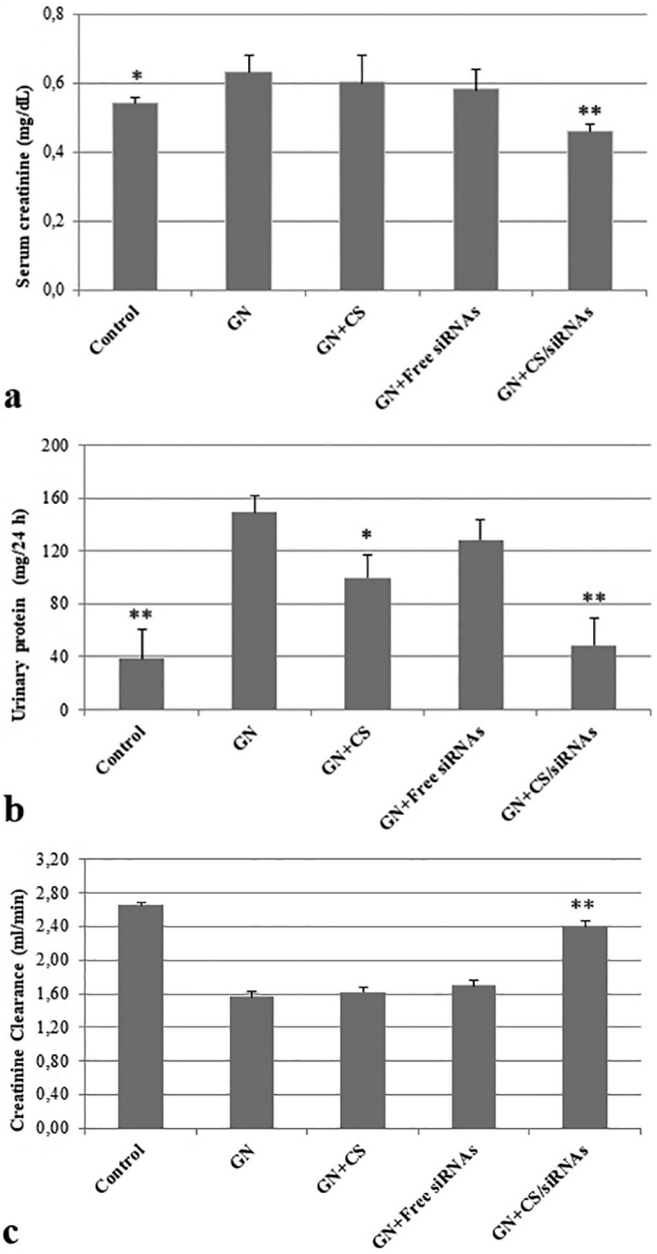

Fig. 2. Blood and urine biochemistry results after 10-day repeated administrations of chitosan, free siRNAs and chitosan/siPDGF-B + siPDGFR-β nanoplexes in Thy-1 glomerulonephritic rats. a. Serum creatinine, b. Urinary protein, c. Creatinine clearence, Values are means ± SD. *$p < .05$ and **$p < .01$ *vs.* GN group.

was 10,6 ± 1,5 mV and the particle size was 307 ± 3,9 nm.

3.2. In vivo studies

3.2.1. The effect of chitosan/siRNA nanoplexes on creatinine and proteinuria

The serum and 24-hour urine samples of groups were collected and proteinuria and creatinine levels in urine and creatinine levels in serum were measured. Serum creatinine levels were 0,54 ± 0,02 mg/dL in the control group, 0,63 ± 0,05 mg/dL in the glomerulonephritis (GN) group and 0,46 ± 0,02 mg/dL in the chitosan/siPDGF-B + siPDGFR-β nanoplexes group. In the GN group, serum creatinine level was found to be higher than control group ($p < 0,05$). In the group given nanoplexes, serum creatinine level was decreased compared with the GN

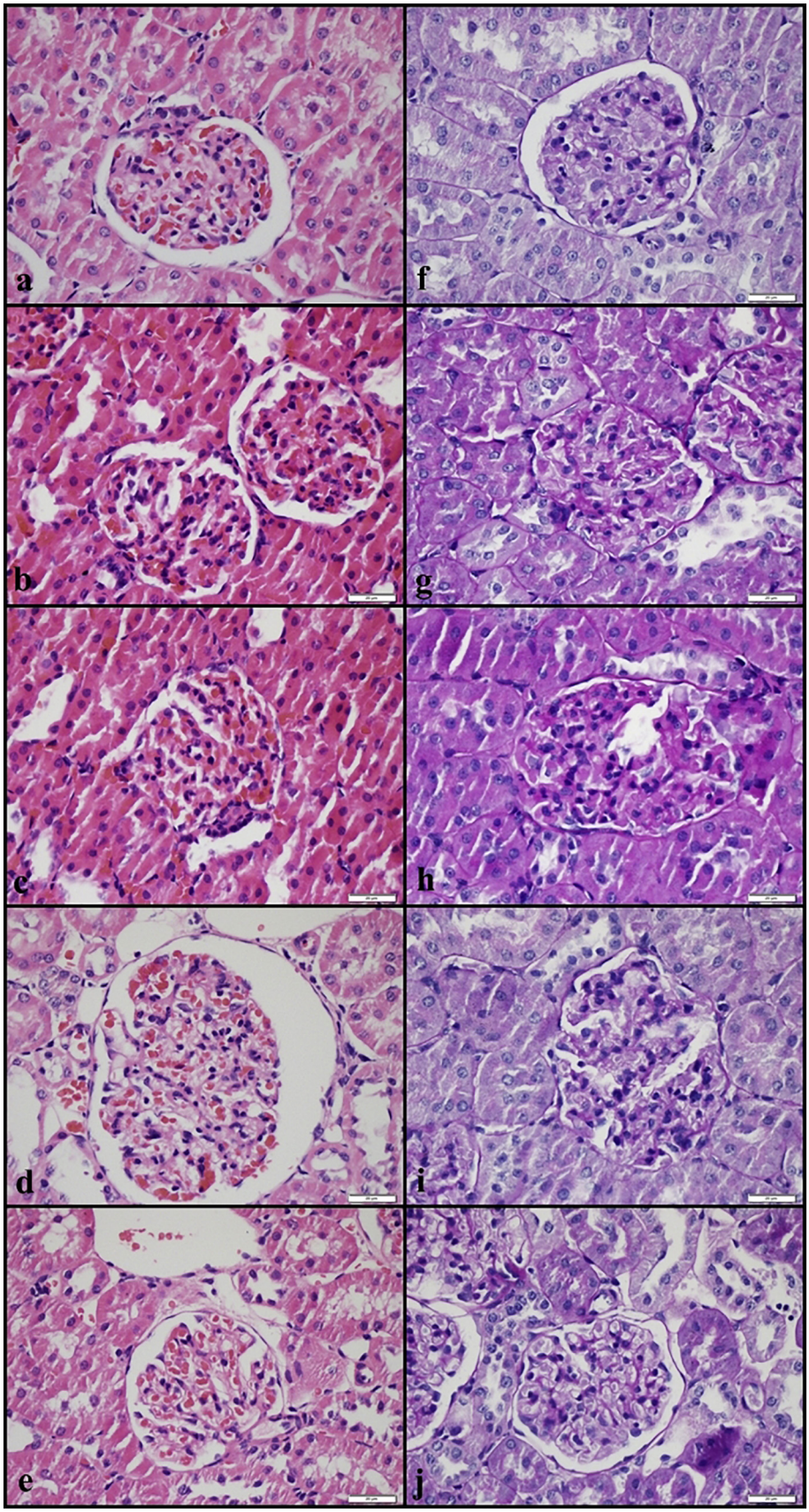

Fig. 3. Nanoplexes administration: effect on glomerular histopathologic changes. a–e: hematoxylin and eosin (HE), f–j:periodic acid-schiff (PAS) stainings. Kidneys of control rats did not shown any alterations (a,f). Thy-1-GN rats that were not treated with chitosan nanoplexes after induction, showing (b) mesangial cell proliferation by H&E and (g) mesangial matrix accumulation by PAS. The administration of chitosan (c, h) and free siRNAs (d, i) were little improvement in renal morphology. Higher improvement in renal morphology was observed in group that administered chitosan/siPDGF-B + siPDGFR-β nanoplexes (e, j).

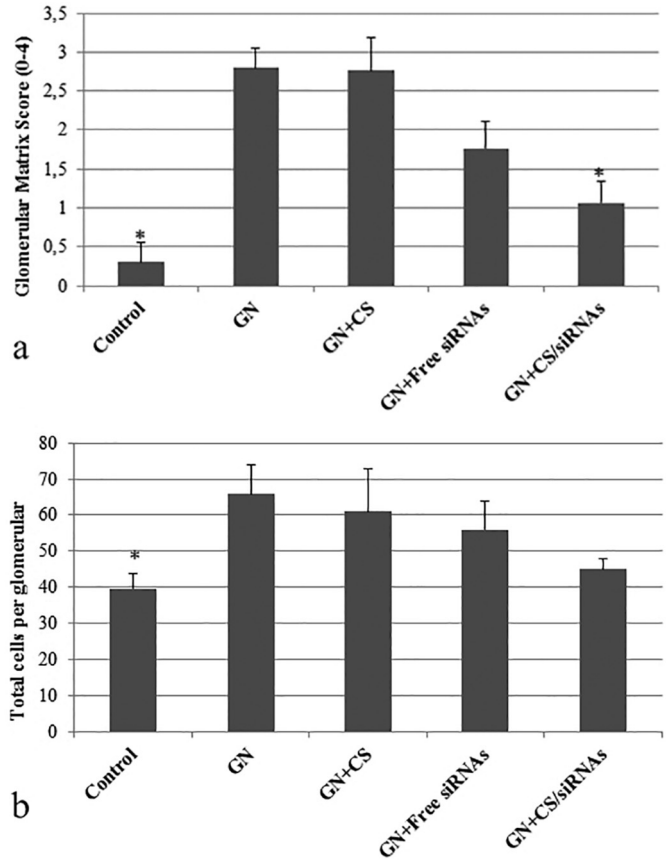

Fig. 4. Chitosan/siPDGF-B + siPDGFR-β nanoplexes reduce proliferation of mesangial matrix accumulation and cell proliferation in Thy-1 glomerulonephritis. a. PAS staining score of each group. A total of 30 glomeruli per rat were scored and averaged for PAS staining. b. The total number of glomerular section of each group.*$p < 0{,}05$ *vs.* GN group.

group. There was a statistically significant difference between nanoplex and GN groups ($p < 0{,}01$) (Fig. 2A). When creatinine and proteinuria values in urine were compared between the groups, there was a significant increase in GN group compared to the control group ($p < .01$). The urinary protein (mg/24 h) level in the group given nanoplexes decreased significantly compared to the GN group ($p < .01$) (Fig. 2B). Creatinine clearance ratio (Ccr) level is an important indicator in determining renal function. Ccr level significantly increased in the nanoplexes-treated group of the Thy-1 GN rats (Fig. 2C).

3.3. Effect of chitosan nanoplexes on glomerular histological changes

Kidney tissues containing at least 30 glomeruli were examined. Histomorphologically, increased cellular proliferation in glomeruli, thickening in basal membranes, and increase in mesangial matrix by H &E and PAS staining have been considered as indicators of glomerulonephritis. The mesangial cell proliferation and matrix deposition in control, glomerulonephritis and treatment groups were shown by H&E and PAS staining, respectively (Figs. 3 and 4). While glomerulonephritis group exhibited a significant increase in cellularity and mesangial matrix, rats treated with chitosan/siPDGF-B + siPDGFR-β nanoplexes showed a decrease in cell proliferation and matrix expansion.

3.4. The determination of PDGF-B and PDGFR-β expressions by immunohistochemistry

Immunohistochemical staining in the kidney tissues were generally observed in mesangial cells, podocyte cytoplasm and extensions, and vascular endothelial and smooth muscle cell cytoplasm. According to the semiquantitative evaluation of PDGF-B staining, 0,5 ± 0,2 in the control group; 3.0 ± 0.5 in the GN group; 2.8 ± 0.2 in the GN + chitosan group; 2,1 ± 0,4 in the GN + free siRNAs group and 1,2 ± 0,1 in GN + chitosan/siPDGF-B + siPDGFR-β nanoplex group. Intensive PDGF-B staining was observed in the mesangial cell cytoplasm in the GN group (Fig. 5B). GN + chitosan/siPDGF-B + siPDGFR-β nanoplex group showed a marked decrease in staining intensity in mesangial cell cytoplasm, and some mesangial cell cytoplasm showed no staining (Fig. 5E).

The semiquantitative evaluation of PDGFR-β staining was 0,9 ± 0,5 in the control group; 3.0 ± 0.6 in the GN group; 2.1 ± 0.3 in the GN + chitosan group; 1,8 ± 0,2 in the GN + free siRNAs group and 1 ± 0,5 in GN + chitosan/siPDGF-B + siPDGFR-β nanoplex group. When PDGFR-β immunoexpression in kidney tissues was evaluated, the highest expression was observed in the GN group (Fig. 5G). In the GN + chitosan/siPDGF-B + siPDGFR-β nanoplex group, a significant decrease in the intensity of PDGFR-β immunostaining was observed (Fig. 5J). In the control group, minimal immunoexpression was observed (Fig. 5F).

3.5. The effect of treatment on apoptosis

In TUNEL study, apoptotic cell staining was not observed in any glomeruli of the control group. A small number of apoptotic cells were seen in the tubules of the control group (Fig. 6). In the glomerulonephritic group, there was a significant increase in the number of apoptotic cells in both glomeruli and tubules (Fig. 6B). There was a decrease in the number of glomerular and tubular apoptotic cells in the nanoplex group (Fig. 6E).

In the glomerular apoptosis scoring, the mean number of apoptotic cells was 0,5 ± 0,2 in control group; 11 ± 1.4 in GN group; 8.5 ± 1.2 in GN + chitosan group; 9 ± 1.5 in GN + free siRNAs group; 2.6 ± 1.5 in GN + chitosan/siRNA nanoplex group. In all groups, it was observed that the number of apoptotic cells was increased compared to the control group. The increase in apoptotic cell number in the GN group was found statistically significant compared to the control group ($p < 0{,}001$).

In the tubular apoptosis scoring, the mean number of apoptotic cells was 9,5 ± 10 in the control group; 90.6 ± 14.3 in the GN group; 65 ± 1.5 in GN + chitosan group; 72 + 6.5 in GN + free siRNAs group and 22.3 + 7.5 in GN + chitosan/siPDGF-B + siPDGFR-β nanoplex group. Compared to the control group, an increase in apoptotic cell number was observed in all groups.

3.6. The determination of PDGF-B and PDGFR-β mRNA levels by qRT-PCR

To investigate the gene silencing effects of chitosan/siRNA nanoplexes, the reduction of PDGF-B and PDGFR-β mRNA was quantitatively measured by qRT-PCR method. qRT-PCR analysis showed that the levels of PDGF-B and PDGFR-β mRNA were significantly decreased in kidney tissues when nanoplexes were administered (Fig. 7). In the chitosan/siRNA nanoplexes group, it was inhibited 65% of PDGF-B expression and 80% of PDGFR-β expression as compared to the glomerulonephritis group. When free siRNAs were administered to kidney, we did not observe any significant decrease in the mRNA levels of PDGF-B and PDGFR-β.

4. Discussion

Mesangial cells, podocytes and endothelial cells within glomerulus play roles in the progression of glomerulonephritic diseases. Therefore, glomerular-targeted therapeutics may be more preferred in therapy of these diseases (Zuckerman and Davis, 2013). Mesangial cells are activated upon the injuries of glomerular basement membrane, podocytes and endothelial cells and these cells contribute to mesangial matrix

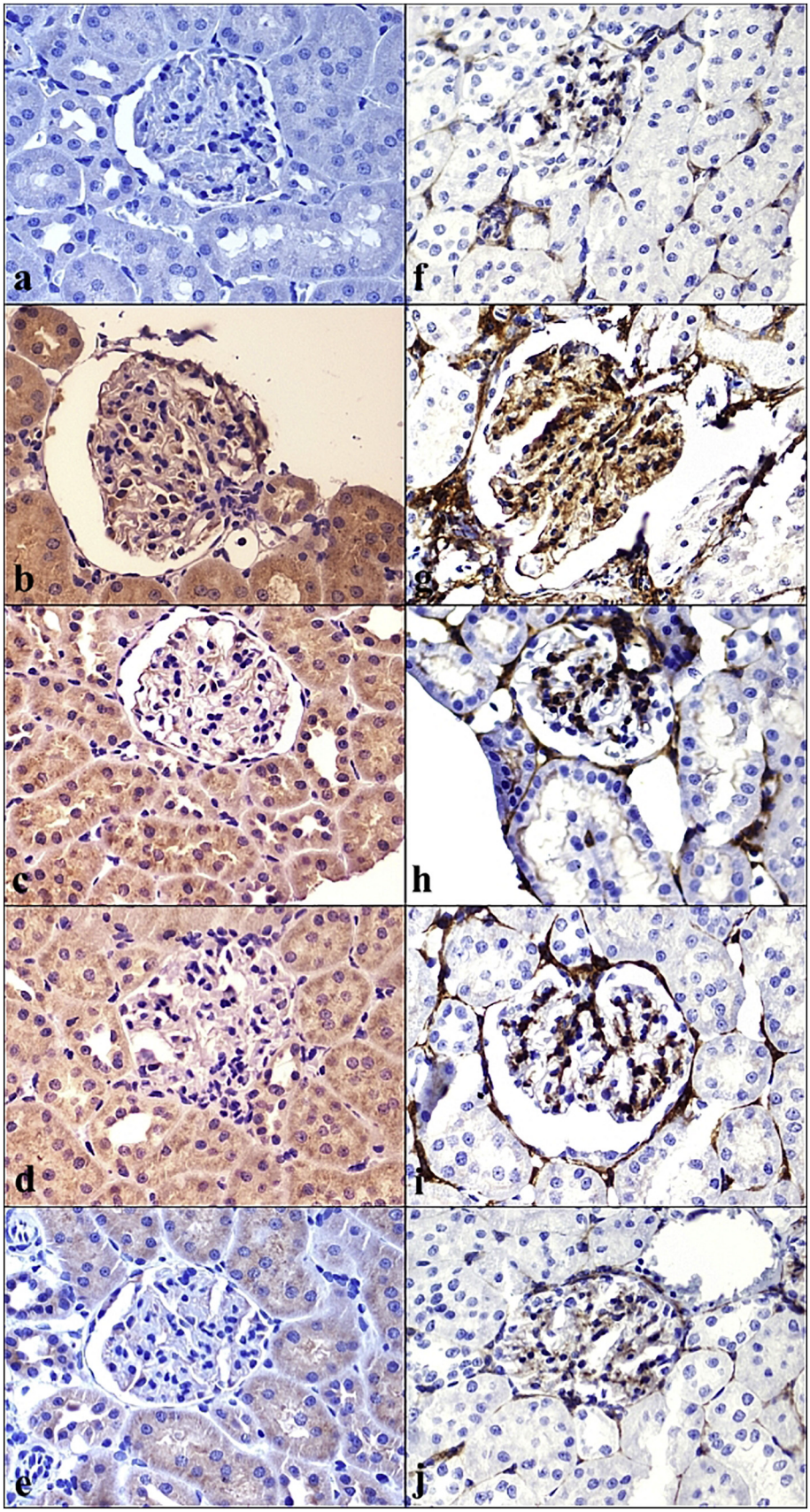

Fig. 5. Immunohistochemical analysis of PDGF-B (a–e) and PDGFR-β (f–j) expressions. Control group (a,f), GN group (b,g), GN + chitosan group (c,h), GN + free siPDGF-B + PDGFR-β (d,i), GN + chitosan/siPDGF-B + siPDGFR-β nanoplexes (e, j). Nanoplexes treatment significantly reduced PDGF-B and PDGFR-β expressions.

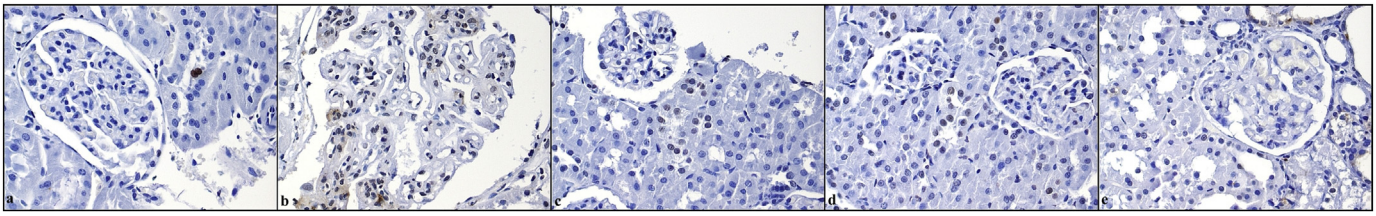

Fig. 6. Detection of apoptotic cells by TUNEL assay. The comparison of glomerular and tubular apoptotic cells among groups (a–e). Control group (a), GN group (b), GN + chitosan group (c), GN + free siPDGF-B + PDGFR-β group (d), GN + chitosan/siPDGF-B + siPDGFR-β nanoplexes group (e).

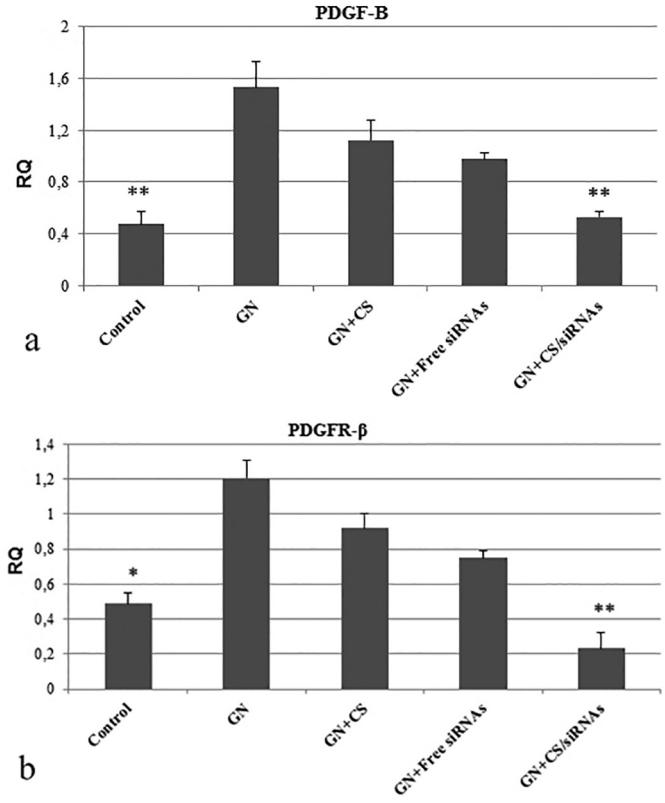

Fig. 7. Effect of chitosan/siPDGF-B + siPDGFR-β nanoplexes on PDGF-B and PDGFR-β mRNA expressions by qRT-PCR assay. *$p < .05$ and **$p < 0.01$vs. GN group.

homeostasis. Mesangial cell proliferation and matrix expansion are biological responses to glomerular diseases or injuries (Abboud, 2012). PDGF-B and its receptor PDGFR-β contribute to mesangial cell development. The overexpressions of PDGF-B and PDGFR-β in glomeruli are related to disease progression of MsPGN. Increased PDGF-B expression in expanded mesangium of nephritic glomeruli was associated with mesangial cell proliferation. Reduction of mesangial cell proliferation and matrix accumulation by blocking signaling pathways in proliferative glomerular diseases is important in the restoration of renal function (Eto et al., 2006). Several studies showed that blocking the PDGF activation pathway was efficient in ameliorating renal disorders such as mesangioproliferative glomerulonephritis, and lupus nephritis (Scindia et al., 2010). In our previous *in vitro* study, we showed that silencing of PDGF-B and PDGFR-β genes by siRNAs reduced mesangial cell proliferation and migration (Şalva et al., 2017). Therefore siRNAs targeting the PDGF-B pathway have been shown to be an effective molecule in the reduction of mesangial cell proliferation *in vitro*.

The RNAi technology used to silence the genes that cause the disease is promising as a new strategy in the treatment of renal disorders. However, siRNAs are quickly removed from the body by the renal blood flow. Therefore, nanocarriers have been developed for tissue or cell-specific delivery of siRNAs. Thus, problems such as toxicity, off-target effects, and unwanted side effects are minimized (Zuckerman et al., 2012). Shimizu et al. (2010) showed that naked siRNAs injected intraperitoneally were eliminated from the blood within 10 min and excreted quickly into the urine. However, siRNAs delivered by the nanocarriers were detected in urine after 1 h and in circulation for 2 h. Gao et al. (2014) suggested tht chitosan/siRNA complexes accumulated in the kidneys after intraperitoneal administration and siRNA level in the kidney remained very high even after 24 h Şalva et al. (2016)) reported strong fluorescence intensity was shown in kidney tissue at 4 h after intravenous injection of chitosan/FITC-siPDGF-B nanoplexes (20/1 ratio). Yuan et al. (2007) showed accumulation of low molecular weight chitosan (LMWC) in the kidneys after intravenous injection. The LMW chitosan can be used for renal targeting as it extends siRNA's blood circulation time and can be specifically taken up by renal tubular cells (Yuan et al., 2007, 2009). Furthermore, glomerular deposition due to increased vascular permeability and inflammation in glomerulonephritis higher than it is in the healthy kidney. Thus, nanoparticle delivery to kidney in glomerular diseases can be increased by this way (Zuckerman and Davis, 2013).

In this study, we used the anti-Thy-1.1 model to create a glomerulonephritis model very similar to the human disease. Thy-1.1 nephritis is a widely used model of proliferative glomerulonephritis (Schaefer et al., 2010). Following the induction of mesangioproliferative anti-Thy-1.1 nephritis, diffuse mesangiolysis and prominent ballooning of capillary loops, severe mesangial proliferation and matrix accumulation occurs (Jefferson, 1999). An abnormal accumulation of the extracellular matrix in the glomerulus is an important biological property of progressive glomerular injury, followed by mesangial cell proliferation in anti-Thy1.1 GN. In our study, mesangial cell proliferation and matrix accumulation in the glomerulonephritis group were observed, and these morphological findings were considered sufficient for the diagnosis of GN. Chitosan nanoplexes containing siPDGF-B and siPDGFR-β were administered intraperitoneally to glomerulonephritis-inducing rats. The important finding of this study was that chitosan/siPDGF-B + siPDGFR-β nanoplexes significantly reduced mesangial cell proliferation and mesangial matrix expansion. Yang et al. (2015) demonstrated that when chitosan/COX-2-siRNA nanoparticles were given intraperitoneally for treatment of kidney injury in obstructive nephropathy, obstructed kidney contained higher amount of siRNA. The advantages of intraperitoneal administration of chitosan/siRNA nanoparticles are evasion of degradative effects of serum, increase of accumulation of siRNA in kidney, and increase of therapeutic effect with low-dose siRNA. Chitosan increases the stability, half-life and biodistribution of siRNA and provides reduction of kidney glomerular filtration rate.

Twenty-four-hour urine and serum samples prior to sacrification of GN and treatment groups were collected to determine the effects of PDGF gene silencing in the kidneys. Urinary protein excretion and creatinine levels in serum and urine were measured. In the GN group, creatinine in the urine and serum and proteinuria in urine increased according to the control group. When the effects of chitosan/siPDGFB + siPDGFR-β nanoplexes on proteinuria were examined, there was observed significant decrease in proteinuria and creatinine values in

treatment group as compared to GN group. Gene silencing with chitosan/siPDGFB + siPDGFR-β nanoplexes inhibits both proliferative pathological changes as well as improving renal function in Thy-1 nephritic rats. Zhang et al. (2014) demonstrated lower levels of BUN and creatinine in therapy group when they were given erythropoietin containing chitosan nanoparticles in IgA nephropathy rat model as compared to the control group. In our study, it was noted that in rats that were given Thy-1, subsequent repeated intraperitoneal administrations of naked siPDGFB + siPDGFR-β have reduced the creatinine and proteinuria in urine to an extent. These results are consistent with the results of other studies that use different siRNA targets in Thy-1 induced nephritis. Qiu et al. (2011) showed that glomerular mesangial cell proliferation, matrix production and urinary protein excretion decreased with naked siRNA targeted to thrombospondin-1 (TSP-1) in the Thy1.1 glomerulonephritis model. Qiu et al. (2009) showed that when nephritis was induced in rats by Thy-1, silencing of the Gadd45c gene by naked siRNA inhibited pathological changes and as compared to the control group, decreased the urinary protein excretion.

Furthermore, it was shown when the biochemical parameters in the urine were examined that administration of chitosan alone reduced urine creatinine and proteinuria as compared to the GN group in rat glomerulonephritis model. The chitosan and its derivates are being widely studied for their diverse physiologic and pathologic activities such as their wound healing, anti-ulcer, anti-tumor, anti-microbial, anti-oxidant and anti-hypercholesteremic effects. There are many publications that examine the anti-oxidant effects of chitosan on glycerol-induced acute renal failure and on chronic renal failure induced by nephrectomy in rats (Anraku et al., 2012; Yoon et al., 2008, 2006). Yoon et al. (2011) created paraquat-induced nephrotoxicity in rats and subsequently, investigated the renoprotective effects of chitosan oligosaccharides. It has been detected that chitosan administration upon nephrotoxicity induction reduces BUN and creatine levels in rats at a substantial level as compared to the control group. It has been also reported that in kidneys chitosan has an inhibitory activity on renin and angiotensin I converting enzyme (ACE) and has a stimulatory effect on renal blood flow that leads to improvement in clinical characteristics. These data explain the detected renoprotective effects of chitosan in our study.

The effects of chitosan/siPDGF-B + siPDGFR-β nanoplexes on PDGF-B and PDGFR-β immunostaining in the Thy1.1 glomerulonephritis were examined by immunohistochemical study. In the GN group, the PDGF-B and PDGFR-β immunostaining was found to be quite high, whereas the GN + chitosan/siRNA nanoplex group provided a significant decrease in the intensity of PDGF-B and PDGFR-β immunostaining. Mesangial resolution begins on day 3 after anti-Thy-1 antibody injection. After 7th day, there is a significant increase in glomerular cell number and the mesangial matrix expands significantly (Cañadillas et al., 2010). In comparison with nephritic rats, treatment with the nanoplexes led to a significant reduction of mesangioproliferative changes and PDGF-B and PDGFR-β protein expressions. Ostendorf et al. (2001) reported that oligonucleotide aptamer agonist PDGF-B suppressed mesangial cell proliferation and matrix accumulation in the anti-Thy-1.1 nephritis model.

In our study, in addition to analyzing the effects of chitosan nanoplexes on PDGF-B and PDGFR-β at the protein level in glomerulonephritis, qRT-PCR was conducted to study the effects at mRNA level. The group that chitosan/siRNA nanoplex was administrated upon GN induction showed a statistically significant reduction in both PDGF-B and PDGFR-β mRNA levels. This phenomenon is in parallel with the reduction in protein expression levels. In addition to this, even though the reduction in PDGF-B expression was not at a level to be statistically significant, there was still some amount of PDGF-B reduction in chitosan only group. In many literatures, it has been suggested that chitosan can be renoprotective due to its antioxidant effects (Anraku et al., 2012; Yan et al., 2006; Yoon et al., 2008, 2011). Mune et al. (2002) have put forth that antioxidants suppress the glomerular sclerosis and mesangial cell proliferation in glomerular disease models and thus, treatments involving antioxidants might prove to be useful in stopping progression of renal diseases. Lu et al. (2018) noted that PDGF-B induced proliferation of vascular smooth muscle cells (CSMC) increased reactive oxygen species (ROS) activity and thus, when chicoric acid, an antioxidant agent, was used to suppress ROS signaling pathway, PDGF-B-induced VSMC proliferation has also been suppressed.

Apoptosis contributes to resolution of renal inflammation and provides clearance of inflammatory cells. It is an important process in repair of proliferative glomerular injury. The normalization of glomerular cell number is regulated by apoptosis. Increased number of mesangial cells in glomerulonephritis returns to normal with apoptosis. The subsequent reduction of mesangial cell proliferation induced by anti-Thy1.1 antibody in the rat is a result of this repair mechanism (Qiu et al., 2009). Glomerular apoptosis increases in 5–7 days, peaking at 10–14 days, returning to normal at 28 days (Jefferson, 1999). In this study, apoptosis was evaluated by TUNEL *in situ* hybridization method in all groups. Treatment with the chitosan/siRNA nanoplexes significantly reduced the number of apoptotic cells in comparison with the GN group. This reduction seemed to be due to reduced mesangial cell proliferation.

The interaction among proinflammatory cytokines, ROS and apoptotic factors results in damage to renal tubules by inducing epithelial cell loss and functional loss in interstitial tubules. Additionally, this interaction affects the renal blood flow and leads to structural and functional changes in the microvasculature. Inflammatory glomerular damage is associated with ROS activity. ROS induces an inflammatory response in mesangial cells. Production of oxygen radicals leads to the apoptosis of glomerular cells. ROS is thus involved in the pathophysiology of the glomerular damage. Therefore, using antioxidants would produce a renoprotective effect. We are suggesting that chitosan, which uses as a carrier system in our study, induced decrease in apoptosis, albeit not at a statistically significant level, when used due to its antioxidant effects (Tamay-Cach et al., 2016).

5. Conclusion

In conclusion, in this study, the therapeutic effects of chitosan nanoplexes containing siRNA targeting PDGF-B and PDGFR-β were investigated for the first time in the anti-Thy1.1 antibody-induced glomerulonephritis model at the molecular, biochemical and light microscopic levels. The results show that chitosan/siPDGF-B + siPDGFR-β nanoplexes can be used as a novel therapeutic strategy in glomerular diseases.

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