Effect of periodontitis on fibrosis and macrophage infiltration in kidneys of rats with chronic kidney disease

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ABSTRACT

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Chronic kidney disease (CKD) presents continuous abnormalities in structure and function of the kidney. Its representative symptoms are elevated blood urea nitrogen (BUN) and creatinine (Cre) levels. Inflammation is one of the factors that induces morphological changes in CKD. Periodontitis is a bacteria-induced inflammatory disease characterized by alveolar bone loss. Bacteria and bacteria-induced substances that are localized in the periodontal tissues may enter the system via blood vessels, and have deleterious effects on distant organs. In clinical studies, there are ongoing studies regarding the association between periodontitis and CKD. However, the mechanisms related to the association between the two diseases have not been clearly elucidated. Researches using animal models of periodontitis and CKD are useful in understanding not only the association, but also the related mechanisms between CKD and periodontitis. Therefore, this study investigated the effects of periodontitis on kidneys in rats with or without nephrectomy (Nx)-induced CKD.

Rats were divided into sham surgery group (Sham group), sham surgery group with tooth ligation (ShamL group), Nx group (Nx group), and Nx group with tooth ligation (NxL group). Two-steps sham and 4/6Nx surgeries were conducted on weeks 5 and 6 of age rats. To confirm the induction of CKD, levels of plasma BUN and Cre of the Sham and Nx groups were evaluated at 16 weeks of age. At 16 weeks,

periodontitis was induced by ligating the mandibular first molars with dental floss. Four weeks after ligation, mandibles of the Sham, ShamL and NxL groups were extracted. To determine successful induction of periodontitis, alveolar bone area and osteoclast numbers (No.) were measured using Hematoxylin and Eosin (H&E) and TRAP stains, respectively. To determine the effects of periodontitis on kidneys, kidneys were extracted at 20 weeks of age. Histopathological changes in renal corpuscles were analyzed using H&E and Jones stains. Tubulointerstitial fibrosis was analyzed using Masson trichrome stain. In addition, macrophage infiltrations and TNFα expressions were evaluated using immunohistochemistry.

The Nx group showed higher plasma BUN and Cre levels than the Sham group. While the ShamL and NxL groups both showed reduced alveolar bone areas and increased osteoclast numbers than the Sham group, there were no differences between both groups. In kidney, the Nx group showed decreased glomerulus No. and increased Bowman's capsule membrane (BCM) thickness, tubulointerstitial fibrosis, and macrophage infiltration compared to those of Sham group. The NxL group had reduced glomerulus No. compared to the Nx group, whereas there were no differences between the Sham and ShamL groups. Only the NxL group showed reduced glomerular area

ratio relative to the Sham group. Interestingly, the ligated groups had increased tubulointerstitial fibrosis and macrophage infiltration than the non-ligated groups. In addition, only the NxL group had elevated TNFα expressions in both renal corpuscle and tubulointerstitium compared to the Sham group.

In the mandibles, CKD did not affect alveolar bone loss by periodontitis. In the kidneys, periodontitis induced increase in tubulointerstitial fibrosis and macrophage infiltration in both cases of absence and presence of CKD. Additionally, periodontitis altered glomerulus No., glomerulus area ratio, and TNFα expression only when CKD was present. These results suggest that periodontitis induce morphological changes in the kidney, but these changes are further exacerbated in the presence of CKD. In addition, macrophage infiltration and TNFα expression in the presence of periodontitis may be associated with the morphological alterations in the kidney.

Keyword: Periodontitis, Chronic Kidney Disease, Macrophage, Fibrosis

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I. INTRODUCTION

Periodontitis is periodontopathogen-induced chronic inflammatory disease that causes disruption to the ecological symbiosis, and is characterized by local

destruction of gingiva, formation of periodontal pockets, and alveolar bone resorption, which could eventually lead to tooth loss[1]. In periodontitis patients, the host responds to the periodontopathogens by activating inflammatory cells. The accumulation of such inflammatory cells leads to periodontal tissue destruction including alveolar bone due to excessive expression of pro-inflammatory mediators, such as TNF α, and IL-1 at the site of infection[2]. Locally produced pro-inflammatory cytokines at inflamed periodontal tissue can enter the systemic circulation, thus, trigger elevated levels of inflammatory markers including C-reactive protein[3]. Studies have shown that periodontopathogens may disseminate through gingival ulceration to distant organs, such as lung, heart, and the placenta[4-9]. Periodontitis-associated microorganisms or cytokines subvert the host's immune homeostasis at their distant locations[3, 10]. Therefore, the relationship between periodontitis and distant organs have begun to be considered in the clinical field.

The kidney is an organ that plays a major role in maintaining the body's homeostasis. It cleans the blood by removing waste products, regulates blood pressure by releasing hormones, maintains red blood cell levels, and promotes healthy bones by producing active forms of vitamin D. To execute such roles, the kidney consists of functional units, nephrons. The nephron is divided into two parts: the renal corpuscle and the renal tubule. The renal corpuscle consists of a mass of

glomerular capillaries (glomerulus), which is encapsulated by the Bowman's capsule. For blood to be filtered, it needs to pass through the walls of glomerular capillaries, basement membrane and filtration slits of the Bowman's capsule. Then, the filtrates flow through the renal tubules where the required minerals are actively re-absorbed back into the blood[11]. Chronic kidney disease (CKD) is defined by consistent deterioration of renal structure or function. It has been recognized as a leading public health issue, and global estimation of prevalence is 13.4% (11.7-15.1%)[12]. CKD has one or more of the following symptoms persisting for over three months; decreased glomerular filtration rate (GFR) to less than 60 mL/min/1.73 m^2, albuminuria of more than 30 mg per 24 hours, or markers of kidney damage. As CKD progresses, elevated blood urea nitrogen (BUN) and creatinine (Cre) and renal morphological changes appear[13]. Nephron loss owing to injury or donation can have hypertrophic effect on the remnant kidneys[13-15]. The remaining nephrons grow in size in order to maintain the homeostasis[13]. Hyperfiltration of nephron leads to podocyte detachment, glomerulosclerosis, and nephron atrophy, which consequently reduce nephron number (No.)[14-17]. In attempt to heal the damaged nephrons, infiltrating immune cells promote the secretion of pro-inflammatory and pro-fibrotic mediators in kidney, thus, exacerbate interstitial inflammation and fibrosis[18].

There are ongoing clinical studies regarding the association between periodontitis and CKD[19, 20]. A 10% increase in periodontal inflammation resulted in 3% decrease in renal function of stage 3-5 CKD patients[21]. Moreover, periodontitis patients had 2.8 times and 3.4 times higher risk of being in stages 4 and 5 of CKD, respectively[21]. Non-surgical periodontal treatments have shown lower risk of end-stage renal disease in CKD patients, thus, act as preventative measures against kidney decline[22]. However, the mechanisms related to the association between the two diseases have not been clearly elucidated.

Animal experiments may be utilized to elucidate the association between the two diseases. Until now, there has been studies on the influence of periodontitis on kidneys of animals with normal condition or systemic diseases. In normal rats, periodontitis increased Bowman's capsular space and degenerated the tubules, and such aggravations have been associated with oxidative stress and lipid peroxidation[23]. Regarding effect of periodontitis on diseased kidneys, previous literatures using animal model with systemic diseases, such as hypertension and obesity, reported that periodontitis can alter the morphology of kidney[24, 25]. However, there have been few animal studies of the effects of periodontitis on kidneys with CKD. As CKD models, IgA nephropathy, unilateral ureteral obstructions (UUO), nephrectomy (Nx), and genetic modifications are utilized[26]. In

an *in vivo* study using the UUO model, it was reported that UUO-induced renal fibrosis were not exacerbated by periodontitis[27]. Comparing the effects of periodontitis on the kidney with normal function or with diverse CKD conditions can provide useful information on the association between the two diseases. Thus, the current study investigated changes in inflammation-related cytokine associated with the morphological alterations due to periodontitis in the kidneys of rats with periodontitis alone or with periodontitis and Nx.

II. MATERIALS AND METHODS

1. Animals and groups

Male Sprague Dawley (SD) rats with sham surgery or Nx were purchased from Shizuoka Laboratory Center (Shizuoka, Japan). Rats were acclimated for a week and maintained in a specific pathogen-free environment with a 12:12 hours light-dark cycle, temperature of 22℃, and humidity of 60%. Rats were divided into four groups: rats with sham surgery (Sham group, n=6), rats with sham surgery and tooth ligation (ShamL group, n=6), rats with Nx (Nx group, n=7), and rats with Nx and tooth ligation (NxL group, n=8). The induction of CKD was assessed in blood of the Sham and Nx groups at 16 weeks of age. Periodontitis induction was assessed in mandibles of the Sham, ShamL, and NxL groups at 20 weeks of age. The effect of periodontitis on kidney was assessed in kidneys of the Sham, ShamL, Nx, and NxL groups at 20 weeks of age. Weights and diets were measured weekly during the experimental period. All experimental protocols

were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC approval No 2019-0245) of Yonsei University.

2. Assessment of CKD induction

2.1 Plasma biochemistry

The Nx group underwent two steps of Nx surgery (Figure 1). At 5 weeks of age, 40% of the left kidney was excised; at 6 weeks of age, the entire right kidneys were completely excised. The Sham group received two steps dorsal incision surgeries. At 16 weeks of age, blood from the lateral tail veins were collected from the Sham and Nx groups for plasma biochemistry analysis (Figure 1). All rats were anesthetized by intraperitoneal injection of 1:1 ratio of Zoletil 50 (30 mg/kg; Virbac, France) and Rompun (10 mg/kg; Bayer Korea, Ansan, Korea) prior to blood extraction. 500 μL of blood was obtained from the lateral tail vein using 24 G $\frac{3}{4}''$ catheter, then were collected in EDTA tubes (BD Bioscience, New Jersey, US). To isolate the plasma, blood was centrifuged at 3000 ×g for 20 minutes. BUN and Cre levels were

measured using FUJI DRI-CHEM 4000i Chemistry Analyzer (Fuji Film, Tokyo, Japan).

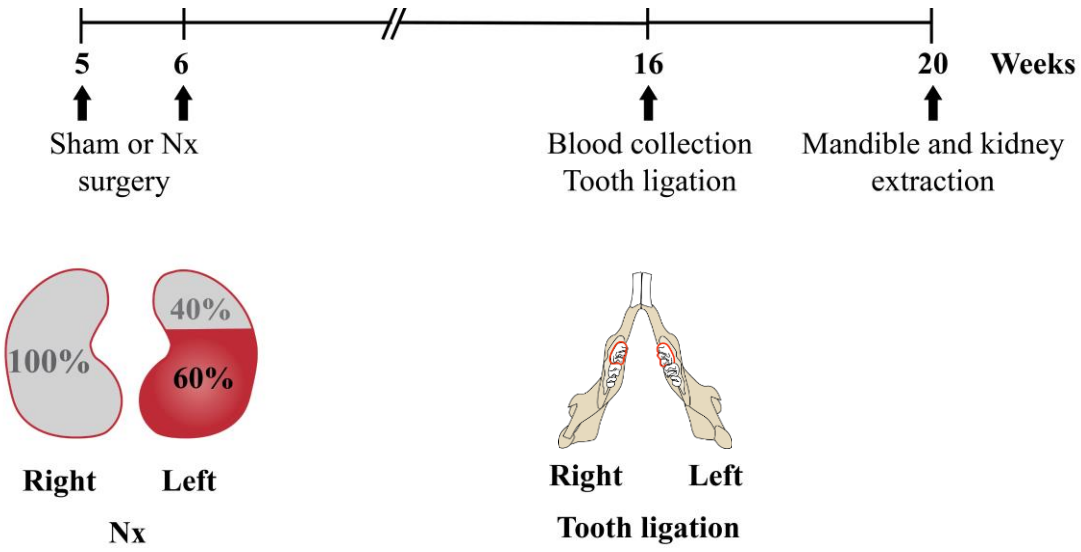

Figure 1. Experimental scheme to assess the influence of periodontitis on kidneys in the presence or absence of CKD. Rats were divided into four groups: sham surgery (Sham group), sham surgery and tooth ligation (ShamL group), Nx surgery (Nx group), and Nx surgery and tooth ligation (NxL group). At 16 weeks of age, blood of all groups were collected, then the ShamL and NxL groups underwent tooth ligation. At 20 weeks of age, mandibles and kidneys were extracted. Nephrectomy; Nx.

3. Assessment of periodontitis induction

3.1 Ligation

At 16 weeks of age, the ShamL and NxL groups underwent bilateral ligation of mandibular first molars to induce biofilm accumulation (Figure 1). After anesthetization with 1:1 ratio of Zoletil 50 and Rompun, dental floss (Oral-B, Ohio, US) was inserted between first and second mandibular molars and tied so that the knot faced the mesial end of the first molar. Ligation condition was checked weekly.

3.2 Mandible preparation

At 20 weeks of age, the Sham, ShamL, and NxL groups were ethically sacrificed using steady influx of CO_2 in a CO_2 chamber (Figure 1). Mandibles were fixated using 10% neutral-buffered formalin (NBF) for 24 hours, then decalcified using 10% EDTA at room temperature for 3 months. Decalcified mandibles were paraffin-embedded then sectioned to 4 μm thickness. The slides were de-paraffinized with 3 stages of absolute xylene,

rehydrated using graded ethanol prior to staining. Histological analysis was conducted using slide scanner (Aperio AT2, Leica Biosystems, Wetzlar, Germany).

3.3 Alveolar bone area assessment

Alveolar bone areas of mandibular first molars were assessed in furcation using Hematoxylin (MERCK, New Jersey, US) and eosin (Sigma, Missouri, US) (H&E) stain. The region of interest (ROI) in the furcation is the area from furcation top extended down 1.5 mm (Figure 3A). Alveolar bone area percentage is calculated by dividing the alveolar bone area by ROI area.

3.4 Osteoclast assessment

Osteoclast formations of mandibular first molars were assessed in furcation using tartrate-resistant acid phosphatase (TRAP) stain (Wako, Osaka, Japan) according to the manufacturer's protocol. Counterstaining

was done with methyl green (Vector Laboratories, California, US). TRAP-positive osteoclasts were counted along alveolar bone surface of the ROI extended 1.5 mm downwards from the alveolar bone crest (Figure 3B), and the osteoclast No was divided by the alveolar bone length.

4. Histological analysis of kidney

4.1 Kidney preparation

At 20 weeks of age, kidneys were extracted from the Sham, ShamL, Nx, and NxL groups. Kidneys were fixated in 10% NBF for 24 hours, then embedded in paraffin. Paraffin blocks were cut to 4 μm thickness. Slides were de-paraffinized in 3 stages of absolute xylene, and re-hydrated in graded ethanol. Histological analysis was conducted using slide scanner (Aperio AT2, Leica Biosystems, Wetzlar, Germany).

4.2 Renal corpuscle assessment

Quantitative analysis of glomerulus No. in cortex was conducted using H&E stain. Only distinguishable glomeruli were counted. Glomeruli No. was counted in 5 fields for each section at ×200 magnification, then divided by field area. Glomerulus area ratio and Bowman's capsule membrane thickness (BCM) in cortex were quantified using Jones stain according to protocol (ab245883; Abcam, Cambridge, UK). Slides were incubated in silver methenamine solution at 65℃ for 45 minutes. Counterstaining was done using nuclear fast red for 10 minutes. The assessments were evaluated in 30 full-sized glomeruli for each section. The glomerulus area ratio was calculated by dividing glomerulus area by renal corpuscle area (Figure 5A). The BCM thickness was assessed by measuring the area between the outer and inner lines of the BCM (Figure 5A).

4.3 Tubulointerstitial fibrosis assessment

Tubulointerstitial fibrosis was evaluated in cortex with fewer than three glomeruli using Masson trichrome stain according to protocol (BBC Biochemical, Texas, US; Sigma-Aldrich, Missouri, US). Tubulointerstitial fibrosis was evaluated in 10 fields at ×100 magnification for each section. The fibrosis, which is visualized in blue, was quantified by the optical density using image processing software, Aperio Imagescope (v12.3.3.5048; Leica Biosystems, Wetzlar, Germany). Interstitial fibrosis percentage was calculated by dividing positive optical density by field area.

4.4 TNFα and ED1 assessment

TNFα expression in glomerulus and tubulointerstitium of the renal cortex was measured using immunohistochemical stain. Slides were incubated in 3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. Incubation in trypsin at 37℃ for 10 minutes was conducted for antigen retrieval. Normal horse serum blocking solution (2.5%; Vector Labs, California, US) for 1 hour at room temperature

was used to reduce non-specific bindings. Rabbit anti-rat TNFα primary antibody (1:175; ab6671; Abcam, Cambridge, UK) was used to incubate slides overnight at 4℃. Slides were incubated in anti-rabbit/mouse IgG conjugated to horse radish peroxidase (HRP) (MP-7500; Vector Labs, California, US) for 30 minutes. The sections were developed using chromogenic substrate, diaminobenzidine (DAB; DAKO, Santa Clara, US). Slides were counterstained with methyl green (R&D systems, Minneapolis, US) for 4 hours at room temperature. TNFα expression was analyzed by optical density in glomerulus and tubulointerstitium, separately (Aperio ImageScope). For glomerulus analysis, 20 glomeruli were selected; for tubulointerstitium analysis, 10 regions at $\times 200$ magnifications were selected.

For evaluation of ED1-positive cell infiltration, endogenous peroxidase of sections was blocked in the same manner as mentioned before. For antigen retrieval, slides were incubated for 20 minutes at 80-85℃ maintained citrate buffer (10 mM, pH 6.0). Then blocked in the same manner using normal horse serum blocking solution. Mouse anti-rat ED1 primary antibody (1:150; MCA341R, Bio-Rad Laboratories, California, US)

was used to incubate slides overnight at 4℃. Slides were incubated with anti-rabbit/mouse IgG conjugated to HRP as secondary antibody for 30 minutes. Slides were developed by dropping DAB substrate, then counterstained with Mayer's Hematoxylin (Sigma-Aldrich, Missouri, US). For ED1 expression, optical densities in 10 fields of tubulointerstitium were analyzed at ×400 magnification (Aperio ImageScope).

5. Statistical analysis

Kruskal-Wallis statistical analysis was conducted, values of $P < 0.05$ were considered significant. Significant data were further analyzed using Mann-Whitney test. Regarding BUN and Cre analysis, $P < 0.05$ were considered significant. Regarding alveolar bone and osteoclast No, $P < 0.017$ were considered significant. For rest of the data, $P < 0.0083$ were considered significant. All data were recorded as means ± standard error (SEM). All data were processed through IBM SPSS Statistics 25 (New York, US).

III. RESULTS

1. Changes in plasma BUN and Cre levels due to Nx

To assess successful establishment of CKD due to Nx, plasma BUN and Cre levels from the Sham and Nx groups were analyzed at 16 weeks of age (Figure 2). The Nx group had significantly elevated BUN level compared to the Sham group (Figure 2A). Similarly, the Nx group had significantly higher Cre level than that of the Sham group (Figure 2B).

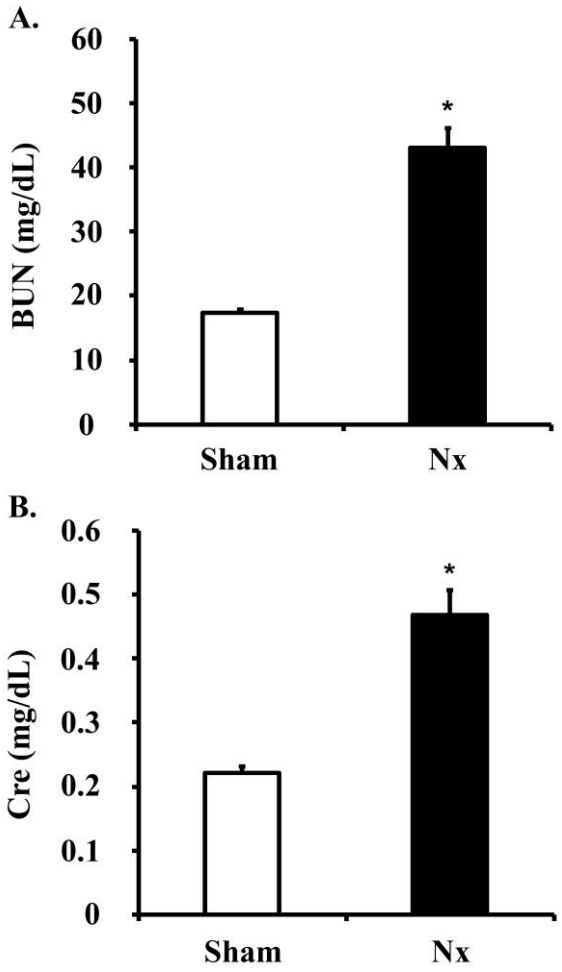

Figure 2. Changes in plasma BUN and Cre levels due to Nx. (A) BUN levels. (B) Cre levels. Data measured by plasma biochemistry analysis. Data are expressed as means ± SEM. * $P < 0.05$. Blood urea nitrogen; BUN, Creatinine; Cre.

2. Changes of alveolar bone area and osteoclast No. by periodontitis

To assess successful establishment of periodontitis by ligation, alveolar bone area and osteoclast No were analyzed in the Sham, ShamL, and NxL groups at 20 weeks of age (Figure 3). The ShamL and NxL groups had significantly lower alveolar bone area percentage than that of the Sham group (Figure 3A). There was no significant difference between alveolar bone areas of the ShamL and NxL groups. TRAP-positive osteoclasts were also counted (Figure 3B). The ShamL and NxL groups showed significantly increase of TRAP-positive osteoclasts compared to that of the Sham group. The osteoclast No. in the ShamL and NxL groups showed no significant difference.

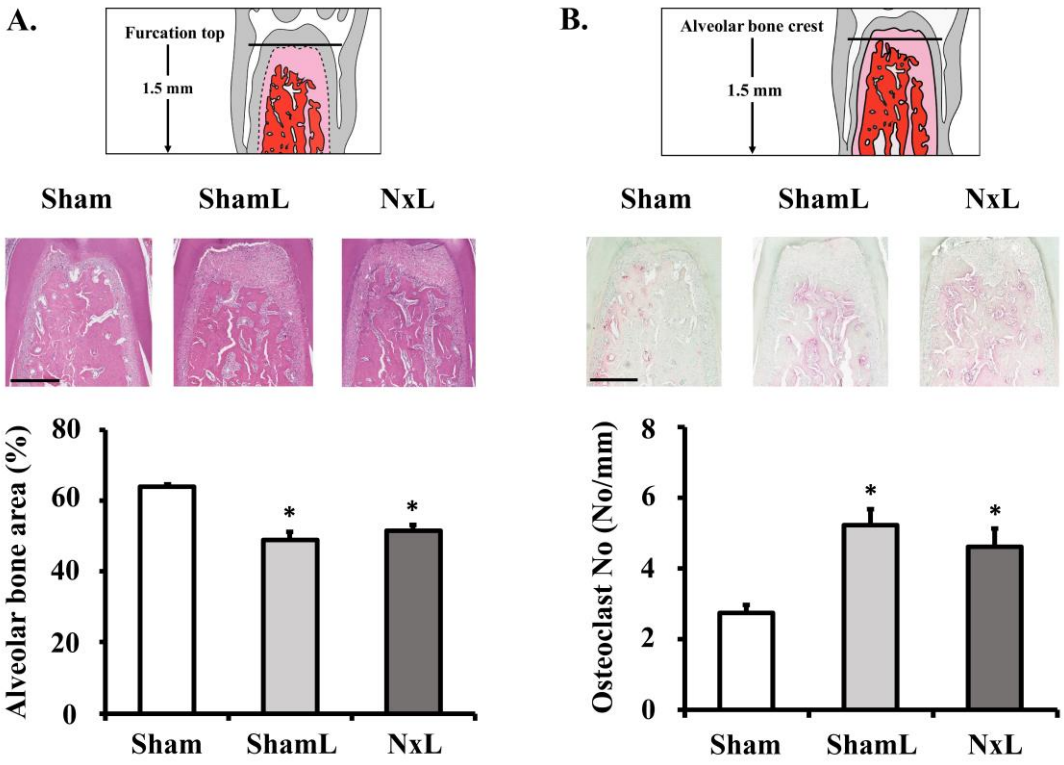

Figure 3. Changes of alveolar bone area and osteoclast No. by periodontitis. **(**A) Alveolar bone area in furcation of mandibular first molar. Upper panel indicates the region of interest (ROI, black dotted line) defined by 1.5 mm vertically dropped from the furcation top. The red within the ROI indicate alveolar bone area. The percentage is calculated by the alveolar bone area divided by the ROI area. Middle panels are representative images of each group (H&E stain). Lower panel represents quantified alveolar bone area. (B) Osteoclast No. in the furcation. Upper

panel indicates the ROI defined by the bone surface from alveolar bone crest downward 1.5 mm. The osteoclasts that are present along the bone surface of the ROI were counted. Middle panels are representative images of each group (TRAP stain). Lower panel represents osteoclast No. Data are expressed as means ± SEM. * $P < 0.017$: versus Sham group. No.; number. Scale bar, 500 μm.

3. Changes in glomeruli No. by periodontitis in the presence or absence of CKD

To observe the effect of periodontitis on renal corpuscle in CKD present or absent conditions, No. of glomeruli were counted at 20 weeks of age (Figure 4). The Nx and NxL groups had significantly less glomerulus No. than the Sham and ShamL groups. Furthermore, glomerulus No. of the NxL group was significantly lower than that of the Nx group (Figure 4B). The ShamL group had similar levels of glomerulus No. compared to the Sham group.

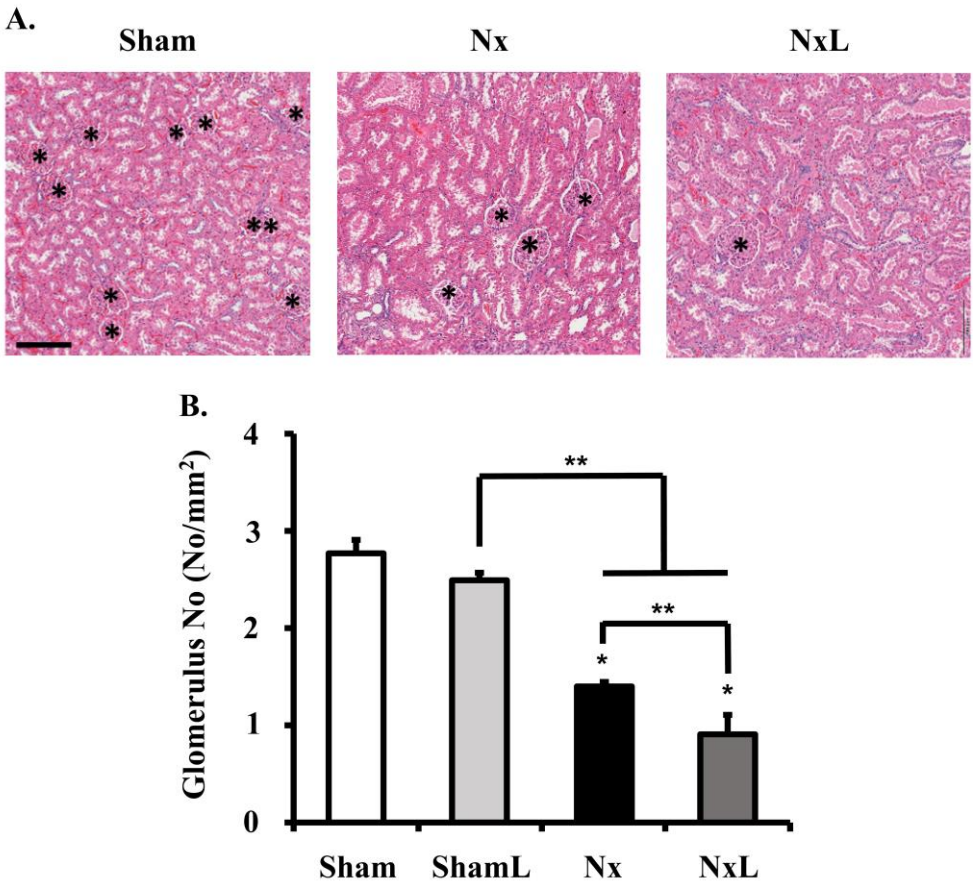

Figure 4. Changes in glomeruli No. by periodontitis in the presence or absence of CKD. (A) The representative images of the Sham, Nx, and NxL groups (H&E stain). Glomeruli are marked with asterisk (*). (B) The glomeruli No.. * P < 0.0083: versus Sham group; ** P < 0.0083: between groups. Scale bar, 200 μm.

4. Changes in structure of renal corpuscle by periodontitis in the presence and absence of CKD

To assess the influence of periodontitis on renal corpuscle structure in the presence and absence of CKD, glomerulus area ratio and BCM thickness were assessed from Jones stain (Figure 5A). In the glomerulus area ratio, only NxL was significantly less than the Sham group among all the groups (Figure 5B). Regarding BCM thickness, it was significantly thicker in the Nx and NxL groups compared to that of Sham and ShamL group (Figure 5C).

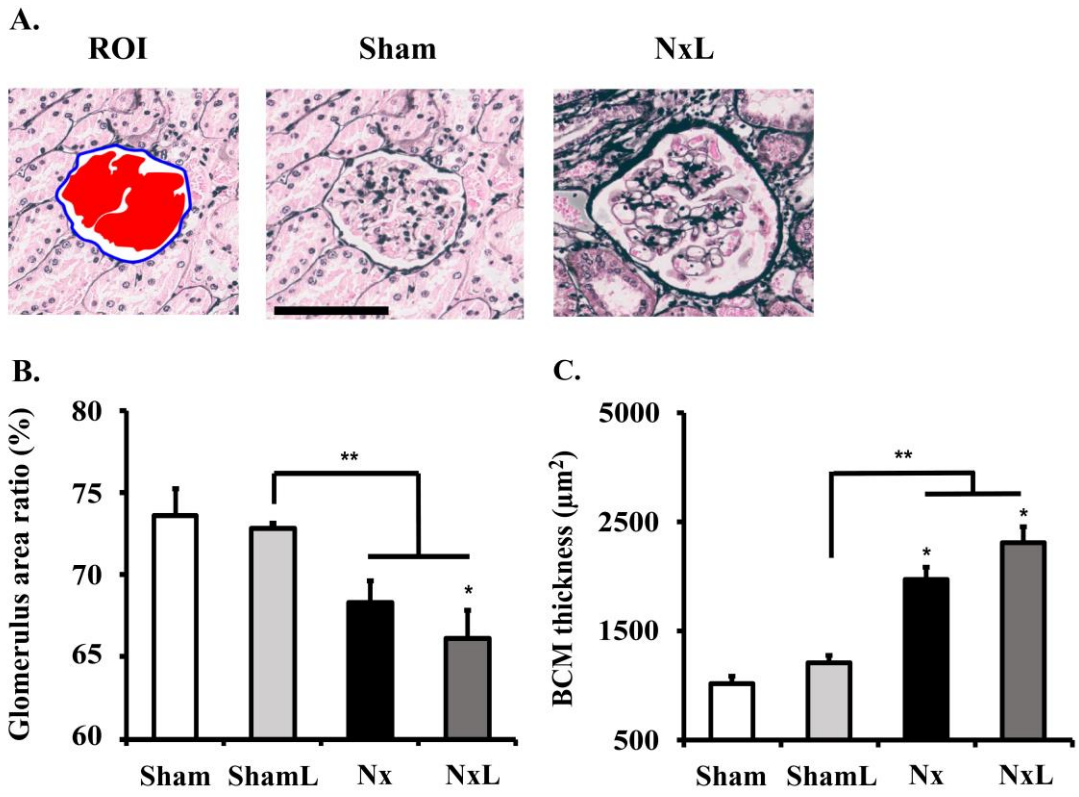

Figure 5. Changes in structure of renal corpuscle by periodontitis in the presence and absence of CKD. (A) ROI and representative images of the Sham and NxL groups. Left panel indicates the ROI of glomerulus are ratio and BCM thickness. Glomerulus area ratio was calculated by dividing the glomerulus area (red) by the renal corpuscle area (blue, white, and red). BCM thickness was measured by the area of the blue line. (B) Glomerulus area ratio. (C) BCM thickness.

Data are expressed as means ± SEM. * P < 0.0083: versus Sham group; ** P < 0.0083: between groups. Scale bar, 100 μm.

5. Changes in tubulointerstitial fibrosis by periodontitis in the presence and absence of CKD

Changes in tubulointerstitium in CKD present or absent conditions by periodontitis was assessed from Masson trichrome stain (Figure 6A). The fibrosis in the ShamL, Nx, and NxL group was greater than that of the Sham group (Figure 6B). The fibrosis in the ShamL and NxL groups was greater than that of the Sham and Nx groups, respectively. When fold changes were compared between ligated and non-ligated groups, there was a 4.35-fold increase in the NxL compared to the Nx group, and a 1.75-fold increase in the ShamL group compared to the Sham group.

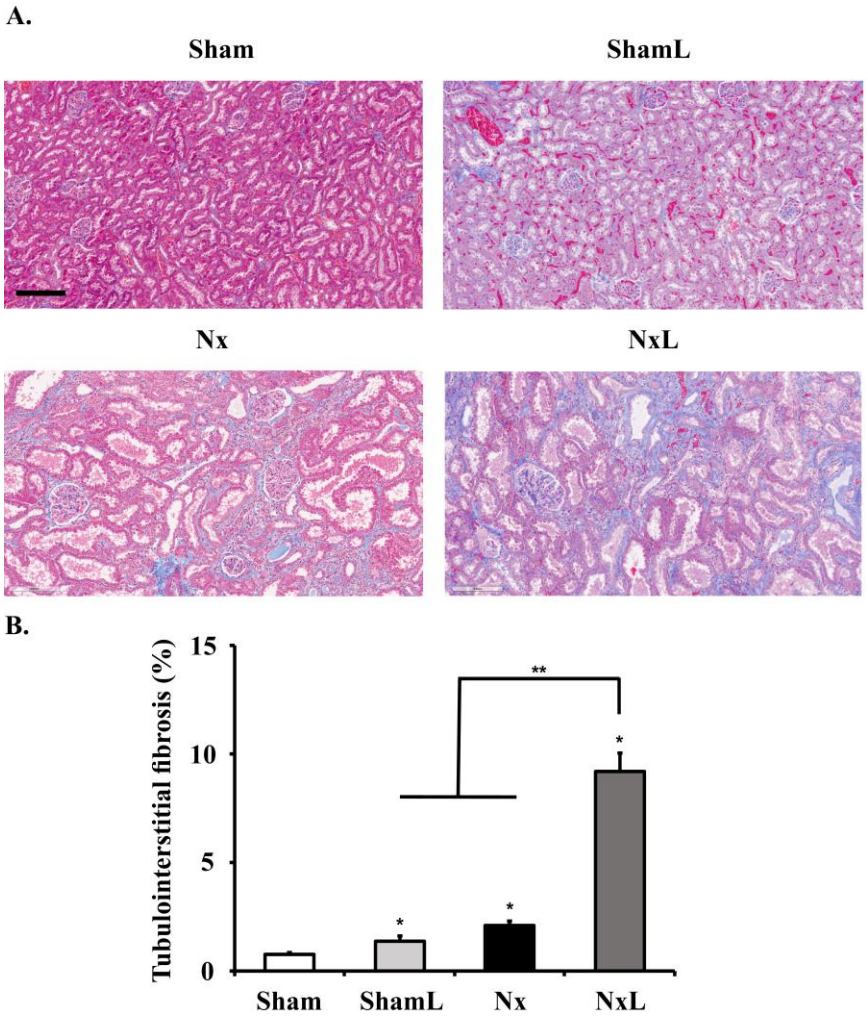

Figure 6. Changes in tubulointerstitial fibrosis by periodontitis in the presence or absence of CKD. (A) Representative images of each group (Masson trichrome stain). (B) Tubulointerstitial fibrosis. Data are expressed as means ± SEM. * P < 0.0083: versus Sham group; ** P < 0.0083 between groups. Scale bar, 200 μm.

6. Changes in TNFα expression of renal corpuscle by periodontitis in the presence and absence of CKD

To observe the inflammatory cytokine expression of renal corpuscle by periodontitis in CKD present or absent conditions, TNFα expressions were analyzed using immunohistochemical stain (Figure 7A). Among all the groups, the NxL group was the only group to be significantly greater than the Sham group (Figure 7B).

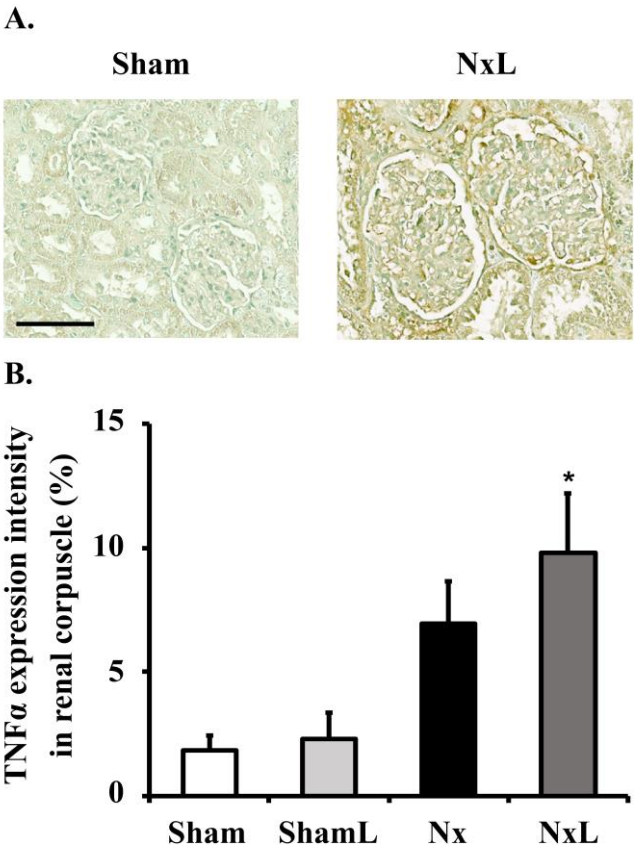

Figure 7. Changes in TNFα expression of renal corpuscle in the presence and absence of CKD. (A) Representative images of the Sham and NxL groups (IHC stain). (B) TNFα expression. Data are expressed as means ± SEM. * P < 0.0083: versus Sham group. Scale bar, 100 μm.

7. Changes in TNFα expression of tubulointerstitium by periodontitis in the presence or absence of CKD.

To observe the inflammatory cytokine expression of tubulointerstitium by periodontitis in CKD present or absent conditions, TNFα expressions were analyzed from immunohistochemical stain (Figure 8A). Among all the groups, the NxL group was greater than the Sham and ShamL groups (Figure 8B).

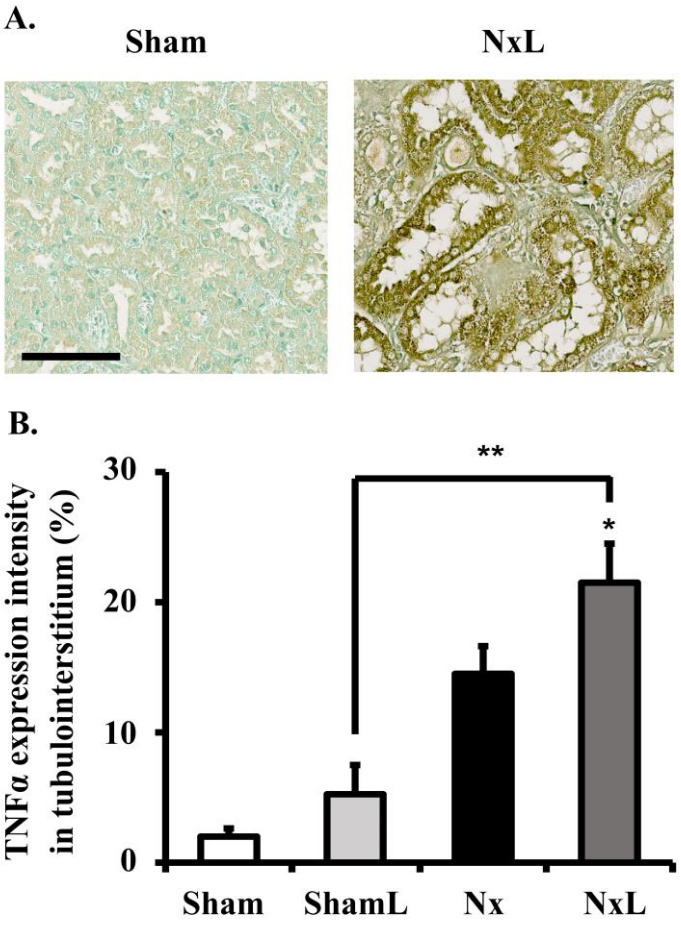

Figure 8. Changes in tubulointerstitial TNFα expression by periodontitis in the presence or absence of CKD. (A) Representative images of the Sham and NxL groups (IHC stain). (B) TNFα expression. Data are expressed as means ± SEM. * P < 0.0083: versus Sham group; ** P < 0.0083: between groups. Scale bar, 100 μm.

8. Changes in interstitial macrophage infiltration by periodontitis in the presence and absence of CKD

To observe the effect of periodontitis on macrophage infiltration in CKD present or absent conditions, ED1 positive macrophage in the tubulointerstitium were quantitatively analyzed (Figure 9). The macrophage infiltration of the ShamL, Nx, and NxL groups was greater than that of the Sham group (Figure 9A and 9B). The ShamL group had significantly higher macrophage infiltration than the Sham group, while the NxL group had significantly higher than the Nx group.

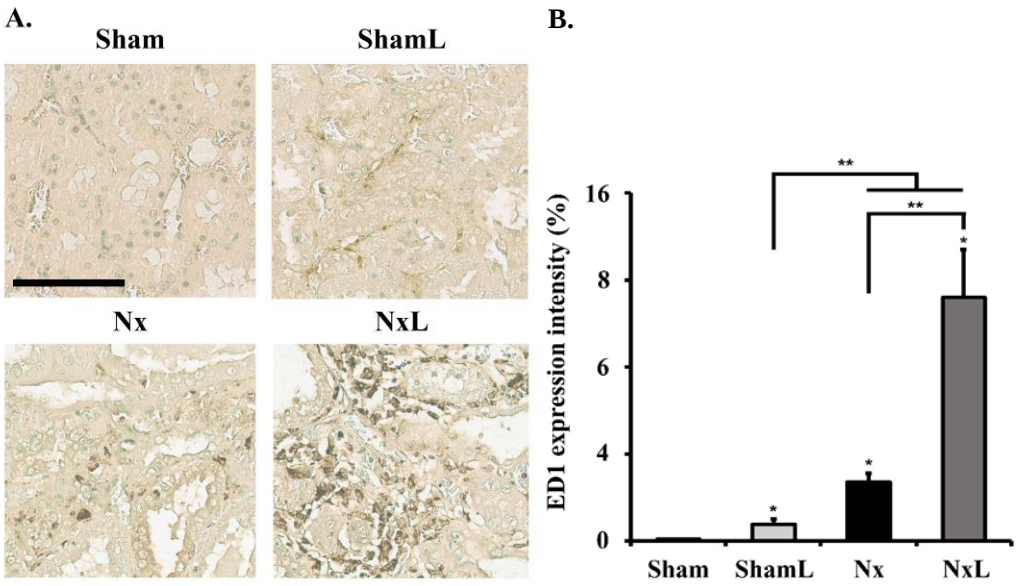

Figure 9. Changes in interstitial macrophage infiltration by periodontitis in the presence or absence of CKD. (A) Representative images of each group (IHC stain). (B) ED1 expression. Data are expressed as means ± SEM. * P < 0.0083: versus Sham group; ** P < 0.0083: between groups. Scale bar, 100 μm.

IV. DICUSSION

Prior to studying the effect of periodontitis on kidneys in the presence and absence of CKD, the study ensured the successful induction of CKD and periodontitis in rats. Abnormal levels of plasma BUN and Cre are the representative symptoms of CKD, which reflect kidney function deterioration to such an extent that waste products in blood are not efficiently filtered in kidney[13]. Prior to tooth ligation, the Nx group had elevated plasma BUN and Cre, thus considered CKD has been successful induced already at this time point. After CKD induction, periodontitis was induced by tooth ligation, which is a common methodology to establish periodontitis model[23-25, 27-29]. After ligation, oral bacteria accumulation along the gingival sulcus cause periodontal inflammation and alveolar bone loss, which are representative symptoms of periodontitis[1, 3]. The present study showed increased alveolar bone loss and osteoclast formation in the ShamL and NxL groups after a month of ligation, which is similar with previously published literatures[30, 31], indicating that periodontitis was successfully induced by ligation. There were no differences in alveolar bone loss and osteoclast formation between ShamL and NxL groups, suggesting that periodontitis symptoms, such as alveolar bone loss and

osteoclast formation, are not affected even if it is accompanied by Nx-induced renal failure.

Brenna et al. reported that reduced nephron number predisposes individuals from developing CKD, thus the close association between glomerulus number and CKD progression have been recognized[32]. The remaining glomerulus undergo a compensatory phenomenon, including exhaustion of glomerular filtration, eventually resulting in glomerular hypertrophy and intraglomerular hypertension[33, 34]. Glomerular exhaustion due to hyperfiltration leads to podocyte detachment, glomerulosclerosis, and nephron atrophy. Therefore, damaged kidney enter a vicious cycle of hyperfiltration that leads to further reduction in glomeruli number[13, 35]. Present data showed that the Nx group had decreased number of glomeruli compared to the Sham group due to CKD induction. Interestingly, the NxL group had significantly less glomeruli number than the Nx group, whereas the ShamL and Sham groups did not exhibit such pattern. This means that periodontitis significantly affects the number of glomeruli in the presence of CKD. Another study, done by Ruta et al, demonstrated the increased sensitivity of kidney with low glomerulus number to secondary insults, which is high salt diet[36]. Genetic modified mice with one kidney did not exhibit glomerulosclerosis, interstitial fibrosis, or

tubular dilatation[36]. However, when challenged with high salt diets, the genetic modified mice developed these altertations[36]. In present study, periodontitis-induced glomerular reduction occurred only in CKD condition may be related to the phenomenon in which Nx-induced glomerular reduction makes kidney more sensitive to secondary challenge, such as periodontitis.

Decreased glomerular numbers result in overexertion of remaining nephron. The remaining glomeruli compensate by increasing glomerular filtration rate, which leads to glomerular hypertrophy[37, 38]. Glomerular hypertrophy is an associated secondary factor to CKD progression, and observed as an increase in area of renal corpuscle and glomerulus[39]. In this study as well, increases in renal corpuscle and glomerular areas were observed in the Nx and NxL groups, but not in the ShamL group (data not shown). There was no difference between the Nx and NxL groups (data not shown). Yang et al. showed that periodontitis group did not increase glomerular area[24], which is in corroboration with our data. However, dissimilar to our data, Yang et al reported that periodontitis and hypertension group had significantly larger glomerulus compared to the hypertension group[24]. It was reported that glomerular area ratio is another indicator to detect abnormalities in the glomerulus[40]. Therefore, the ratio of glomerular area to renal corpuscle area

(glomerular area ratio) were compared in this study. Only the NxL group had a significantly smaller glomerular area ratio compared to the Sham group. BCM thickening is another marker that indicates structural changes in the renal corpuscle. A study from Holderied et al. reported that BCM thickened in diabetic nephropathy conditions by increased expressions of collagen type IV, collagen type I, and TGF-β, which are hallmarks of sclerosis[41]. According to the data, the Nx and NxL groups had thicker BCM relative to the Sham and ShamL groups. The BCM thickeness observed in Nx and NxL groups may be due to Nx rather than ligation as there were no differences between ligated and non-ligated counterparts. Taken together, data suggests when CKD is accompanied by periodontitis, the glomerulus structure is adversely affected.

Renal fibrosis is a histological change of CKD progression, in which the hallmark is excessive accumulation of extracellular matrix (ECM)[17]. Tubulointerstitial fibrosis is characterized by deposition of ECM between the tubules and peritubular capillaries, and is deposited mostly by fibroblasts and myofibroblasts[17, 42]. Tubulointerstitial fibrosis leads to impairment of oxygen and nutrition supply to the tubular cells, which can eventually lead to tubular atrophy and fibrosis aggravation[43]. Mice models with more severe form of

systemic diseases that causes renal dysfunction, such as obesity[25] and hypertension[24], indicated that periodontitis aggravates renal fibrosis. The current study showed the ShamL and NxL groups had greater tubulointerstitial fibrosis than their non-periodontal groups, which are Sham and Nx groups, respectively. When fold changes were compared between ligated and non-ligated groups, there was a 4.35-fold increase in the NxL compared to the Nx group, and a 1.75-fold increase in the ShamL group compared to the Sham group. These results suggest that even when periodontitis is present alone, it can adversely affect the kidneys and that the adverse effects of periodontitis can be stronger in the presence of CKD. Result from previous study reporting that periodontitis did not increase renal fibrosis in the kidneys of normal mice and UUO mice is different from the present results[27]. Since the differences may appear depending on the animal species, periodontitis induction period, and CKD induction method, it is necessary to confirm such points in future studies.

Degree of macrophage infiltration is closely associated with extent of CKD severity and renal fibrosis[44, 45]. The current study looked at the association between macrophage infiltration and periodontitis-induced exacerbation of renal fibrosis. Periodontitis increased macrophage infiltration in both the NxL and ShamL group

compared to the Nx and Sham groups, respectively. Macrophage is a versatile player in renal inflammation and fibrosis as they can acquire different phenotypes after activation: while classically activated M1 macrophage plays an active role in host defense by producing pro-inflammatory molecules, such as IL-1β and TNFα, the alternatively activated M2 macrophage can directly promote renal fibrosis by profibrotic factor production, such as TGF-β and MMP-9[46]. Production of TGF-β can induce proliferation of fibroblasts, and activate fibroblasts into myofibroblasts for excessive ECM production. Previous literature has shown monocytes undergo macrophage-myofibroblast transition (MMT) in UUO mice via TGF- β[47]. MMP-9 production by macrophages can also induce formation of myofibroblast via tubular epithelial-mesenchymal transition (EMT) in the tubulointerstitial region[48, 49]. Therefore, macrophage infiltration could be involved in aggravation of tubulointerstitial fibrosis by periodontitis in both presence and absence of CKD.

Persistent inflammation is one of the symptoms of CKD, and a possible mechanism in which periodontitis aggravates renal fibrosis[46, 50]. TNFα is one of the representative pro-inflammatory cytokines[51]. Podocytes, mesangial cells, tubular epithelial cells, and macrophages in kidney can express TNFα[46]. Thus, this study observed the TNFα expression in renal corpuscle and tubulointerstitial regions

separately. In the renal corpuscle, only NxL group had significantly elevated levels of TNFα compared to the Sham group, while in the tubulointerstitial region, only the NxL group had significantly elevated TNFα expression compared to the Sham and ShamL groups. TNFα upregulation induces renal damage by promoting inflammation and caspase-mediated cell death[29, 52, 53]. Furthermore, production of TNFα by tubular epithelial cells potently activate NF-kB, JNK, and p38 pathways, which aggravates renal inflammation and fibrosis[41, 54]. TNFα induces tubular epithelial cells to undergo EMT, thus showing myofibroblast-like morphology and aggravating fibrosis[55]. These data suggests periodontitis may aggravate renal fibrosis and macrophage infiltration via TNFα in the presence of CKD.

In conclusion, periodontitis increased macrophage infiltration and tubulointerstitial fibrosis in the absence of CKD. Particularly, in the presence of CKD, periodontitis increased macrophage infiltration and tubulointerstitial fibrosis, and further aggravated kidney by reduced glomerulus number, reduced glomerulus area ratio, as well as, increased TNFα expression. These data suggest that periodontitis not only induces morphological alterations in normal kidney, but also exacerbates such alterations in CKD conditions. These renal morphological

alterations may be associated with periodontitis-induced inflammatory responses, such as macrophage infiltration and TNFα expression.

V. References

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국문요약

만성신질환 랫드에서 치주염이 신장 섬유화 및 대식세포 침윤에 미치는 영향

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이연수

만성신질환은 신장의 구조와 기능에 지속적인 이상을 나타낸다. 대표적인 증상은 혈액의 크레아티닌 (Creatinine; Cre) 및 혈중요소질소 (Blood urea nitrogen; BUN)의 증가이다. 염증은 신장의 형태적 변화를 유발하는 요인

중 하나이다. 치주염은 세균에 의한 염증질환으로 치조골 소실을 보인다. 치주염 시 치주 조직의 세균 및 세균에 의하여 발현된 물질이 혈관을 통해 이동하여 다른 장기에 영향을 미칠 수 있음이 제시되어 치주염과 만성신질환의 연관성에 대한 임상연구가 이루어지고 있으나 두 질환의 연관성에 대한 기전은 명확히 밝혀져 있지 않다. 따라서, 본 연구에서는 정상 또는 신장을 적출한 신질환 랫드 모델에서 치주염이 신장에 미치는 영향을 평가하였다.

랫드를 대조군 (Sham), 치주염군 (ShamL), 신질환군 (Nx) 및 신질환 동반 치주염군 (NxL)으로 나누어 실험을 진행하였다. 5 주령 및 6 주령에 신장을 적출한 신질환 랫드를 사용하였으며, 16 주령에 Sham 및 Nx 군의 BUN 및 Cre 을 측정하여 신질환 유발을 확인하였다. 치주염은 16 주령에 하악 제 1 대구치를 결찰하여 유도하였으며, 20 주령 (결찰 4 주 후)에 Sham, ShamL 및 NxL 군의 하악을 채취하여 치주염 유발 여부를 평가하였다. 치주염 유발은 Hematoxylin & Eosin (H&E)과 TRAP 염색을 통해 치조골 소실과 파골세포 형성을 측정하여 평가하였다. 치주염이 신장에 미치는 조직병리학적 변화를 평가하기 위해 20 주령에 신장을 채취하였다. H&E 염색으로 사구체 수, Jones 염색으로 Bowman's capsule membrane (BCM)의 두께 및 소체 대비 사구체 비율을 평가하였고, Masson trichrome 염색으로 세뇨관 간질 섬유화를 평가하였다. 또한 면역화학염색으로 신장의 대식세포 침윤과 TNF α의 발현을 평가하였다.

Nx 군은 Sham 군보다 높은 BUN 및 Cre 수치를 보였다. ShamL 및 NxL 군의 치조골 면적은 Sham 군 보다 감소하였고, 파골세포형성은 증가하였으나 두 군 간에는 차이가 없었다. 신장에서 Nx 군의 사구체 수는 Sham 군에 비해 감소하였고, BCM 의 두께, 세뇨관 간질 섬유화 및 대식세포 침윤은 증가하였다. 사구체 수는 NxL 군에서 Nx 군에 비해 감소하는 반면, Sham 군과 ShamL 군 사이에는 차이를 보이지 않았다. 소체 대비 사구체 비율에서는 NxL 군만이 Sham 군에 비해 감소를 보였다. 흥미롭게도, 치주염 유발 군들의 세뇨관 간질 섬유화 및 대식세포 침윤은 치주염을 유발하지 않은 군들 보다 증가하였다. 또한 NxL 군은 신장 소체와 세뇨관 간질 모두에서 Sham 군에 비해 높은 TNFα 발현을 보였으나, 다른 군들은 Sham 과 차이를 보이지 않았다.

신질환은 치주염에 의한 치조골 소실에 영향을 미치지 않았다. 치주염은 신질환 존재 여부와 상관 없이 모든 경우에 신장의 세뇨관 간질 섬유화 및 대식세포 침윤을 증가시켰다. 치주염은 신질환이 존재하는 경우에만 사구체 수, 소체 대비 사구체 비율 및 TNFα 발현에 변화를 유도하였다. 이러한 결과는 치주염이 신장의 형태적 변화를 유도하며, 치주염에 의한 형태학적 변화는 신질환이 존재하는 경우 더욱 악화될 수 있음을 나타낸다. 또한, 이는 치주염에 의한 대식세포 침윤 및 TNFα 발현 증가가 신장의 형태학적 변화와 관련이 있을 수 있음을 시사한다.