Original Article

Cyclophosphamide modulates Gsk 3β/NF-KB p65 SIgnaling in MRL/lpr mice with lupus nephritis

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Abstract:

Aim: The objective is to investigate the importance of determining the expression of Gsk 3β/NF-κB in MRL/lpr mice and to evaluate the role of cyclophosphamide on this expression.

Methods: For the time gradient, a total of 30 female MRL/lpr mice at 4 weeks old were used and 30 female C57BL/6 mice were used for each of the time groups that is 12, 16, 20, 24 and 28 weeks. A total of twelve female MRL/lpr mice were used in the drug intervention assigned to the CTX group and SLE lupus nephritis (LN) group. In the meantime, six female C57BL/6 mice were used as the normal control (C57). Beginning at 16 weeks of age, the mice in the CTX group received weekly intraperitoneal injections of 1.8 mg of CTX, while both the LN group and the C57 group were given an equivalent volume (0.1 mL) of saline.After a 12-week treatment period, samples were collected. The levels of Gsk 3β and NF-κB p65 in serum and urine were evaluated via ELISA, as well as the gene and protein expression levels of Gsk 3β and NF-κB p65 in kidney tissues, which were analyzed using rt-qPCR and Western blotting techniques.

Results: Time gradient: Development of the LN model in MRL/lpr mice: At 16 weeks of age, elevated concentrations of 24-h urinary protein, antinuclear antibodies (ANA), and double-stranded DNA (dsDNA) were observed in the LN group in contrast to the C57 control group. Pathological assessments and immunofluorescence analysis of the kidneys in LN group mice revealed glomerular cell proliferation, segmental lesions, crescent formations, tubular degeneration, interstitial fibrosis, and infiltration of inflammatory cells. Granular or lumpy deposits of IgA, IgG, IgM, C1q, and C3 were detected within the glomeruli. Compared to the C57 group mice, the expression levels of Gsk 3β and NF-κB p65 in the kidney tissues from the LN group were significantly higher across all age categories ($p < 0.05$). Regarding pharmacological intervention, the 24-h urinary protein levels, ANA, and dsDNA in the CTX treatment group were

significantly reduced in comparison to the LN group of mice ($p < 0.05$), and the pathological changes and the amount of immune complex deposition were also significantly reduced. In the kidneys of the group treated with CTX, the expressions of the genes and proteins for Gsk 3β and NF-κB p65 were notably decreased compared to the LN group ($p < 0.05$).

Conclusion: In mice with LN, increased amounts of Gsk 3β and NF-κB p65 genes and proteins may contribute to the progression of kidney damage. Targeting the downregulation of these genes and proteins in the renal tissue of LN mice may serve as a pharmacological aim for treatment with CTX. According to a recent study, CTX was found to inhibit the activation of Gsk 3β and NF-κB p65 in MRL/lpr mouse kidneys. This is the first time evidence of the potential protective mechanism of CTX was provided.

Keywords: Lupus nephritis, Gsk 3β, NF-κB p65, MRL/lpr mice, cyclophosphamide.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune condition that affects many bodily systems. The exact cause is unknown, but it is mainly due to the failure of immune tolerance to self-antigens, which leads to increased production of autoantibodies. A build-up of self-antigen/antibody complexes in various organs causes damage to these organs and notable clinical features[1]. The ratio of males to females is highest in the age of reproduction range. Between a quarter and 60% of diagnosed SLE will develop lupus nephritis (LN) within five years of diagnosis, while approximately 10% will progress to end-stage renal disease within ten years of diagnosis of LN[2,3]. The ten-year and the twenty-year survival rates for LN patients are around 70% and 60%[4]. It is therefore of significant importance to illuminate the pathogenic mechanisms concerning LN to assess viable therapeutic approaches. Therefore, exploring biomarkers that can distinguish LN activity and its severity, predict renal pathological changes, monitor treatment response, and track disease progression is currently a critically

needed research direction.

The predominant type of NF-κB activation is p65, which is consistently found in the B lymphocytes of lupus patients during their active phase, and this activation is linked to the prognosis of LN[5]. In individuals diagnosed with LN, an upregulation of NF-κB is observed in the mesangial cells of the glomerulus as well as in the mesangial matrix, accompanied by an elevation in inflammatory cytokines. Glycogen synthase kinase 3β (Gsk 3β) acts as a multifunctional serine/threonine kinase that plays a critical role in the Wnt signaling pathway, functioning as an important upstream regulatory protein.

Research has shown that Gsk 3β acts as a positive regulator in activating NF-κB, playing a critical role in inflammation[6]. Furthermore, Gsk 3 is integral to the differentiation of pathogenic Th17 cells within the lymph nodes[7]. It may also play a role in producing autoantibodies, such as anti-double-stranded DNA (dsDNA) antibodies, and in the onset of glomerulonephritis in MRL/lpr mice[8]. However, despite being an important regulatory signaling protein situated at the intersection of the Wnt and NF-κB signaling pathways, Gsk 3β has not been extensively studied in the context of lupus nephritis, with its precise mechanisms still needing full clarification.

Cyclophosphamide (CTX) is the primary medication for the acute phase treatment of LN. Compared to glucocorticoid (GC) monotherapy, standard-dose CTX extends renal survival time[9]. However, there is limited research on the specific mechanisms of CTX in treating LN.

This study, through time-gradient experiments, provides experimental evidence for determining the timing of drug intervention. The research examines how a 12-week CTX intervention influences the signaling proteins Gsk 3β and NF-κB p65 in MRL/lpr mice. This study not only assesses the signaling pathways influenced by CTX treatment but also explores the molecular mechanisms and targets that CTX regulates in the development of LN.

EXPERIMENTAL ANIMALS AND METHODS.

LN model mice

A total of thirty 4-week-old female MRL/lpr mice, utilized as a LN model, were obtained from Cavens Biogle (Suzhou) Model Animal Research Co., Ltd. These mice were confirmed to have specific pathogen-free (SPF) status and had an average weight of 22.85 ± 1.34 g. In addition, thirty 4-week-old female C57BL/6 mice, also maintaining SPF conditions and weighing 21.62 ± 1.41 g, were sourced from the Experimental Animal Center at Gansu University of Chinese Medicine. All experimental mice were kept in the SPF animal facility located at the same center.

Time gradient: Thirty LN mice and thirty C57 mice were randomly divided into groups of 12, 16, 20, 24, and 28 weeks of age, and raised to the corresponding ages. Drug intervention: Twelve LN mice were randomly divided into the LN-CTX group and the LN group, with an additional six C57 mice serving as the blank control group. The LN-CTX group was given intraperitoneal injections of 1.8 mg CTX weekly, while both the LN and C57 groups received a comparable volume of saline each week during the 12-week intervention period.

At the experimental endpoint, mice were humanely euthanized via intraperitoneal injection of sodium pentobarbital. The serum was collected from blood samples for further analysis and then freeze at -80 ºC. A tissue sample was taken from the kidney and preserved in a 10% neutral buffered formalin solution for fixation for histopathological purposes, and the remaining fraction was immediately frozen in liquid nitrogen for further test purposes. All protocols for animal research were approved by the Medical Ethics Committee of Gansu Provincial People's Hospital under Approval No. 2020-169. All experiments complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

24-h urine protein test.

Upon thawing the urine samples, they were placed in a biochemical analyzer as per the operational standards, which assessed the concentration of urinary protein in mice over 24 h. The amount of protein in the urine was multiplied by the volume of urine collected to determine 24 h urinary protein.

ELISA

(1) Allow the body fluid sample to sit. Thaw the body fluid sample and centrifuge it again. Collect the supernatant for aliquot preparation. Arrange standard wells, sample wells, and blank wells in a proper configuration. The instructions that are labelled (2) Addition of samples and enzymes: Add successively 50 μL with standard dilutions approximately the concentration to standard wells as per the concentration gradient. After that, add 40 μL of sample diluent, followed by 10 μL of testing sample into the wells. Subsequently, introduce 100 μL of enzyme-labeled reagent to every well. Cover the plate and incubate at 37 °C for 60 min. (5) Washing: Dispense the liquid out, wash the wells with washing solution for five times and make them dry. Introduce 50 μL of color reagent A and B each into the solution, and keep the plate at 37 °C in the dark for 15 min. End the experiment: Add 50 μL of the stop solution. 4. Measurement: The OD should be read at wavelength of 450 nm through the microplate reader. Use the OD values from standard wells to build standard curve equation and calculate the concentration value. This value is to be multiplied by 5 for its actual concentration in the calculations.

Pathology and immunofluorescence

Hematoxylin/eosin (HE) staining

(1) First, fix tissue (the sample or specimen) and put it in the dehydrator for 8 h. Next, dip tissue (the sample or specimen) in xylene solutions three times followed by gradient ethanol (10 min each). (2) Embedding: Embed in paraffin, allow to dry on a cold plate, and then remove. (3) Sectioning and selection: Section the paraffin block using a microtome and lay the sections flat on the surface of a slide. (4) Baking and drying: Bake the slides in a slide warmer for 15 min and dry them in a drying oven for 30 min.

(5) Dewaxing: Immerse the slides in three xylene solutions sequentially for 15 min each, then in gradient ethanol sequentially for 5 min each, followed by washing. (6) Staining: Stain with hematoxylin for 2 min and rinse with running water. Immerse in 1% hydrochloric acid alcohol solution for 1 second and rinse with water. Return to blue with 1% ammonia water for 1 min and rinse with water. Stain with eosin for 3 min. (7) Dehydration and clearing: Soak in gradient ethanol for 3 min each, followed by soaking in three changes of xylene for 5 min each. (8) Mounting: Mount with neutral resin. (9) Photography and analysis: Microscopic examination, image acquisition, and photographic analysis.

Periodic acid-silver methenamine (PASM) staining

(1) Dewaxing - Washing: Same as 4.1 (1) - (5). (2) Oxidation: Soak in oxidant for 15 min, then wash. (3) Staining and Water Bath Staining: Iron alum solution for 10 min, then wash; silver hexamine solution for staining in water bath at 60 °C, stop when the section changes color, then wash. (4) Toning: Gold chloride toning for 1-2 min, then wash; hypo for 1 min, then wash. (5) Counterstaining, Dehydration, and Clearing: Light green solution for counterstaining for 1 min, then wash; dehydrate with ethanol, then clear with xylene. (6) Mounting: Mount with neutral resin. (7) Photographing and Analysis.

MASSON staining

(1) Dewaxing to washing: Same as 4.1 (1)-(5). (2) Staining and differentiation: Stain with Weigert's iron hematoxylin for 5-10 min. Wash with water after treating with acid alcohol for 10 seconds. (3) Blueing: Treat with Masson's blueing solution for 3-5 min, then wash with water. (5) Staining: Stain with Ponceau fuchin for 5-10 min. (6) Solution washing: Treat sequentially with weak acid-phosphomolybdic acid-weak acid working solution for 1 min each. (7) Staining, dehydration, and clearing: Stain with aniline blue for 2 min, wash with weak acid working solution, dehydrate with ethanol, and clear with xylene. (8) Mounting: Mount with neutral resin. (9) Imaging and analysis.

Detection of immune complex deposits featuring IgA, IgG, IgM, C1q, and C3 in the renal tissues of mice through an indirect immunofluorescence assay.

(1) After freezing the kidney tissue, cut into 3μm sections and let stand for 30 min. (2) Fix with acetone at 4 °C for 10 min, then wash. (3) Block with fetal bovine serum for 20 min. (4) Add the primary antibody. (5) Incubate at 37 °C for 30 min, then proceed with the washing step. (6) Introduce the secondary antibody that is labeled with a fluorochrome. (7) Same as (5). (8) Mount the slides and observe under a fluorescence microscope.

Reverse transcription real-time quantitative polymerase chain reaction (rt-qPCR)

Extract ribonucleic acid (RNA)

(1) Enzyme removal: All items used in this experiment were soaked in DEPC water overnight and then autoclaved to remove enzymes. (2) Tissue grinding: Take 50mg of renal cortex tissue and place it in an EP tube, pre-cooled with physiological saline at 4 °C, centrifuge at 12,000r/min for 5min, wash three times, mince with ophthalmological scissors, add 600μL of pre-cooled Trizol, and homogenize with a homogenizer for 30s, then place on ice for 5min. (3) Centrifugation: Incorporate 120μL of chloroform, mix thoroughly by shaking, allow it to sit at room temperature for 5 min, and then centrifuge at 4 °C at a speed of 12,000 rpm for 5 min. (4) Supernatant collection: Collect the supernatant in an EP tube, add an equal volume of isopropanol, mix well, and let it act on ice for 10min. (5) Centrifugation: Centrifuge at 4 °C, 12,000r/min for 10min. (6) Remove the supernatant, then incorporate 1 mL of 75% ethanol for washing. (7) Centrifuge the mixture at 12,000 rpm for 5 min at a temperature of 4 °C, then discard the supernatant. Once dried, reconstitute in 10 μL of water that is free from nucleases. (8) Measure concentration: Use a nucleic acid analyzer to measure the RNA concentration. Wipe the device with nuclease-free water three times to zero, then measure the RNA concentration and purity. If the concentration is too high, dilute with nuclease-free water. The purity should be ≥ 1.8 for 260/280.

Reverse transcribed cDNA

(1) Follow the instructions of the kit and sequentially add:

RNA	2μL
Oligo(dT)15	1μL
Random Primer	1μL
Nuclear-Free Water	6μL

(2) Denaturation: Following complete mixing, put the sample into the PCR machine, heat it to 70 °C for a duration of 5 min, and subsequently cool it on ice for another 5 min. (3) Prepare the reverse transcription solution based on the kit's guidelines.

Nuclear-Free Water	1.5μL
GoScriptTM 5X Reaction Buffer	4μL
MgCl2, 25mM	2μL
PCR Nucleotide Mix, 10mM	1μL
Recombinant RNasin Ribonuclease Inhibitor	0.5μL
GoScriptTM Reverse Transcriptase	1μL

(4) Reverse transcription: Following the complete mixing of the components, place them into the PCR machine. Set the temperature to 25 °C for 5 min, followed by 42 °C for 1 h and finally, 70 °C for 15 min in order to complete reverse transcription.

Real-time quantitative PCR

(1) After thawing and centrifuging the GoTaq qPCR Master Mix, place it on ice for later use. (2) Prepare the reaction mixture and aliquot it into the reaction wells.

Upstream primer (10μM) and downstream primer.

Nuclear-Free Water	7μL
Upstream primer (10μM)	0.4μL

Downstream primer(10μM)	0.4μL
GoTaq qPCR Master Mix，2X	10μL
CXR 100X	0.2μL

Primer/Internal Reference Sequence [Table 1].

Table 1. Sequences of upstream and downstream primers for Gsk 3β, NF-κB p65, and the internal reference.

Gene	Upstream Sequence	Downstream Sequence
Gsk 3β	5’ TTCTCGGTACTACAGGGCACCA 3’	5’ GTCCTAGCAACAATTCAGCCAACA 3’
NF-κB p65	5’GAAGAACAAGAAATCCTACCCACA 3’	5’ GGTCAGAATGCACCAGAAGTCC 3’
GAPDH	5’TGTGTCCGTCGTGGATCTGA 3’	5’ TTGCTGTTGAAGTCGCAGGAG3’

(3) Add 2 μL of cDNA template to the corresponding reaction wells, and use 2 μL of nuclease-free water as the negative control. (4) Secure the 8-tube strip and perform a brief centrifugation, then place it in the PCR machine to amplify under the following reaction settings: 10 min at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 min at 60 °C, concluding with a ramp of 14 seconds per degree Celsius from 60 °C to 95 °C. (5) Record the Ct value to calculate the RQ value (relative quantity: relative expression level).

Western blotting (WB)

Extract proteins from kidney tissue

(1) Solution preparation: Prepare the lysis buffer according to the ratio of RIPA lysis buffer and PMSF as specified in the instructions, mix well and set aside. (2) Washing: Place 50mg into an EP tube, centrifuge under the aforementioned conditions with pre-cooled saline for 5 min, wash three times, and mince with ophthalmic scissors. (3)

Homogenization and lysis: Place several small steel beads, add 500μL of lysis buffer, homogenize using a homogenizer, and incubate on ice for 2 h for complete lysis. (4) Centrifugation: Centrifuge the mixture for 10 min under the previously mentioned conditions, then keep the supernatant and place it in a -80 °C ultra-low temperature freezer for storage.

Protein content determination

Follow the guidelines provided by the BCA protein assay kit to determine the protein concentration.

Gel preparation

(1) Preparation: Wash the glass plates with ultrapure water, dry them, and clamp them firmLy in place. (2) Preparing the separating gel: Create a separating gel with a concentration of 10% by following the given guidelines. Once TEMED is included, mix the solution well and pour it into the gel plate. Allow the mixture to rest at room temperature for 30 min to allow it to solidify. (3) Formulating the stacking gel: Prepare a 5% stacking gel according to the instructions, mix thoroughly, and add it to the glass plate. Fill it up and insert the comb. Allow it to rest at room temperature for half an h so that the stacking gel can solidify, after which it should be placed in the electrophoresis buffer and stored in a refrigerator set to 4 °C.

Electrophoresis

(1) Sample Loading: Add electrophoresis buffer to the electrophoresis tank, remove the comb, and load protein samples and Marker into the wells respectively. (2) Electrophoresis: Run at 90V until the red and green bands of the Marker spread out, then increase the voltage to 120V. Once the samples have traveled to the bottom of the separating gel after 60 min, switch off the device.

Membrane transfer

(1) Cut the PVDF membrane and soak it in methanol for 5 min. (2) Fully saturate the

filter paper and sponge pads with the transfer buffer. (3) Layer the transfer cassette by placing the sponge pad first, followed by filter paper, separating gel, PVDF membrane, another layer of filter paper, and finally the sponge pad again. Ensure that the cassette is secured, cover the transfer apparatus, and perform the transfer at 220 mA for 90 min. After completion, turn off the apparatus and remove the PVDF membrane.

Closure and reaction

(1) Solution preparation: Dissolve 0.5g of skimmed milk powder in 10mL of 1X TBST solution, and mix thoroughly. (2) Blocking: Immerse the PVDF membrane in 5% milk solution and block on a shaker for 2 h. (3) Primary antibody: Follow the instructions to dilute the primary antibody, mix it well, and then incubate with the PVDF membrane at 4 °C overnight. (4) Secondary antibody: Rinse the PVDF membrane with TBST for 10 min, repeating this process 3 times; dilute the secondary antibody as per the guidelines, ensure thorough mixing, and then incubate at room temperature for 1 h; subsequently, repeat the washing steps for the membrane.

Exposure imaging

Prepare ECL luminescent solution by mixing A solution and B solution in a 1:1 ratio, and use the gel imaging system according to the instructions for exposure and imaging.

2.7 Immunohistochemistry (IHC)

(1) Deparaffinization and rehydration: Paraffin sections are deparaffinized and rehydrated through three changes of xylene and graded ethanol. (2) Antigen retrieval: Conduct the antigen retrieval process using a sodium citrate buffer at a pH of 6.0 for 3 min at a temperature of 100 °C. (3) Inactivation of endogenous peroxidase activity: Add endogenous peroxidase blocking agent and incubate for 30 min. (4) Blocking of nonspecific sites: Add normal goat serum working solution for blocking and incubate for 10 min. (5) Primary antibody: Prepare a dilution of the primary antibody following the manufacturer's guidelines, then introduce the primary antibody and allow it to incubate overnight at 4 °C. (6) Secondary antibody: Introduce the biotinylated goat

anti-rabbit IgG polymer and let it incubate for 30 min. (7) Add the horseradish peroxidase-labeled streptavidin working solution and incubate for 10 min. After adding the freshly prepared DAB solution for colour development, observe the sections under a microscope. The time for color development should be managed carefully and then, the pieces should be washed with tap water. (9) Counterstaining stain nuclei with hematoxylin staining solution for 8 min. (10) Differentiate, Dehydrate, and Mount: Dehydrate with graded ethanol, clear with xylene, and mount with neutral balsam.

Assessment of staining intensity and positive stained cells of the signalling proteins Gsk 3β and NF-κB p65 in the renal tissue samples was carried out using a semi-quantitative approach. The staining reading was classified as 0 (no colour), 1 (pale yellow), 2 (yellowish brown), and 3 (brown). In addition, the ratio of cells that tested positive was measured. The staining intensity of the stain and the percentage of stained cells positively was multiplied to get the evaluation index.

Statistical analysis

Data that are normally distributed are designated mean ± standard deviation (x ± s) notation. The independent samples *t*-test was used to compare two different groups, while one-way ANOVA was used to compare multiple groups. A *p*-value of less than 0.05 was statistically considered significant. The software SPSS version 26.0 was used in all analyses.

RESULTS

Time gradient

Mice in the LN group were found to have higher levels of 24-h urinary protein and serum autoantibodies

Urinary protein excretion over 24 h and levels of ANA were found to be significantly greater in the LN group compared with those in the C57 mouse group ($p < 0.05$) from 16 to 28 weeks of age. The serum dsDNA levels increased significantly between week 12 and week 28 in the study ($p < 0.01$, Table 2).

Table 2. Levels of 24-h urinary protein and serum autoantibodies in LN group mice and C57 group mice at various weeks of age.

	Group	12W	16W	20W	24W	28W
24-h urine protein (mg)	LN	0.63 ± 0.16	1.43 ± 0.29**	2.39 ± 1.02*	1.99 ± 0.70*	2.13 ± 0.94*
	C57	0.61 ± 0.06	0.73 ± 0.16	0.73 ± 0.28	0.73 ± 0.15	0.75 ± 0.08
T		0.316	4.771	2.406	3.512	3.935
P		0.760	0.001	0.020	0.014	0.011
ANA (ng/mL)	LN	20.52 ± 1.65	20.73 ± 1.00*	23.05 ± 2.77*	25.00 ± 0.84***	25.14 ± 1.17***
	C57	18.90 ± 0.56	19.14 ± 0.52	18.64 ± 0.16	18.84 ± 0.47	19.05 ± 0.68
T		1.957	3.155	3.555	14.284	10.460
P		0.086	0.013	0.023	0.000	0.000
dsDNA (ng/mL)	LN	279.38 ± 10.27**	291.45 ± 21.98**	279.95 ± 13.12**	290.53 ± 10.44***	291.56 ± 18.95***
	C57	230.98 ± 18.36	237.76 ± 12.99	236.95 ± 16.97	236.15 ± 8.69	235.00 ± 11.75
T		5.144	4.702	4.482	8.952	5.596
P		0.001	0.002	0.002	0.000	0.000

*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

The LN group of mice exhibited more severe pathological damage and heavier immune complex deposition

The kidney abnormalities observed in mice from the LN group predominantly demonstrated features characteristic of type III and type IV lupus nephritis. Significant

observations comprised the proliferation of mesangial cells and the mesangial matrix, as well as the accumulation of immune complexes in the mesangial area. Furthermore, indications of glomerular crescent formation, localized tubule atrophy, dispersed inflammatory cell infiltration within the renal interstitium, and a slight thickening of small artery walls were noted [Figure 1].

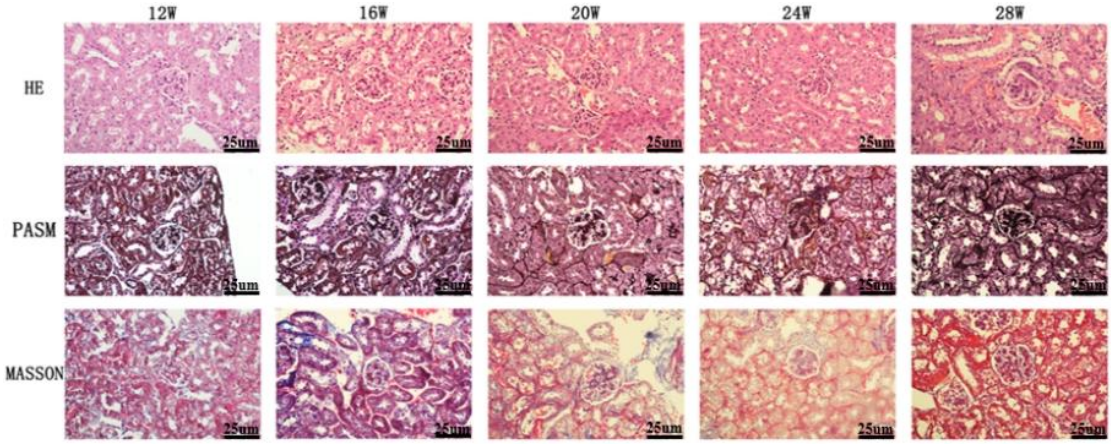

Figure 1. Pathological image of the kidney in LN group mice (40 X).

At every time interval, either granular or lumpy accumulations of IgA, IgG, IgM, C1q, and C3 were detected within the mesangial region of the glomeruli and the capillary walls of mice in the LN group, showing the most pronounced deposition of immune complexes in LN mice at 28 weeks of age [Figure 2].

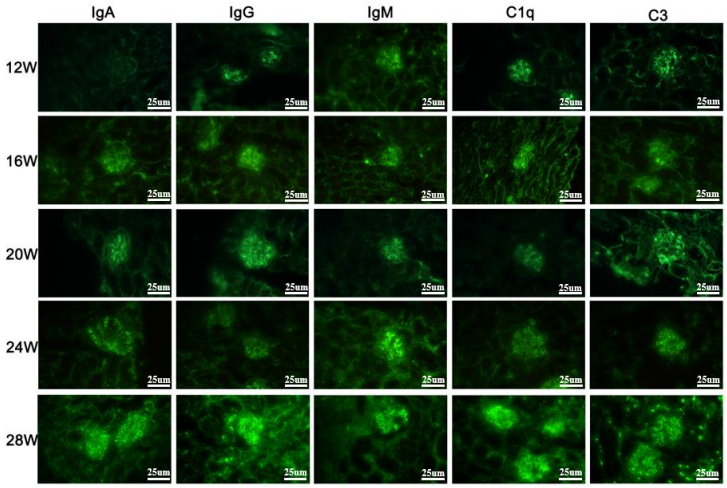

Figure 2. Immunofluorescence image of the kidney in the LN group mice (40 X).

The mouse kidney tissues in the LN group showed increased levels of the signaling proteins Gsk 3β and NF-κB p65

Unlike the C57 group, the LN group displayed increased levels of Gsk 3β and NF-κB p65 in serum and urine; nonetheless, these variations were not statistically significant ($p > 0.05$, Figure 3a-d).

Similarly, the LN group also demonstrated significantly higher gene expression levels of Gsk 3β and NF-κB p65 within kidney tissues between the ages of 12 and 28 weeks ($p > 0.05$, Figure 3e-f).

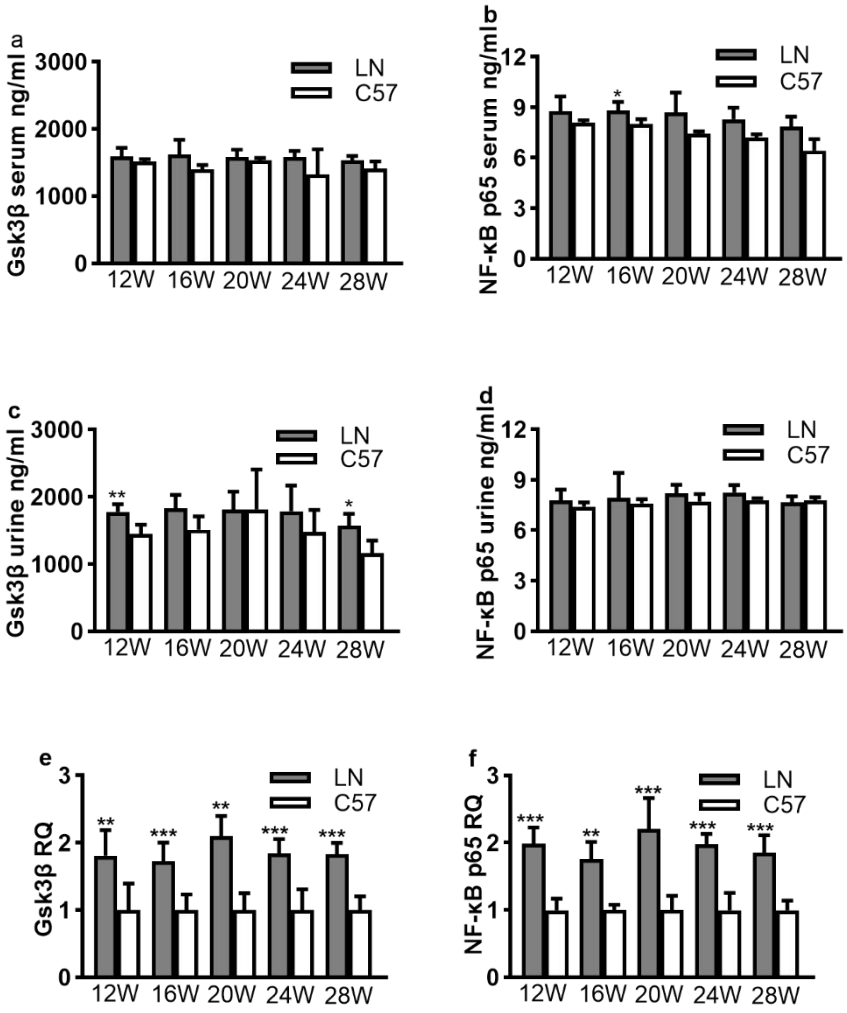

Figure 3. Expression levels of Gsk 3β and NF-κB p65 in serum, urine, and kidneys of mice at various weeks of age. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Pharmacological Intervention.

CTX treatment can reduce autoantibody levels

The urinary protein levels measured over 24 h in the CTX group were found to be lower than those in the LN group; however, this discrepancy did not reach statistical significance ($p > 0.05$, Table 3). In contrast, the CTX group showed significantly lower serum concentrations of ANA and dsDNA antibodies in comparison to the LN group ($p < 0.001$). Moreover, substantial variations in the serum levels of both ANA and dsDNA antibodies were identified among the three groups ($p < 0.001$, Table 3).

Table 3. Levels of 24-h Urinary Protein and Serum Autoantibodies in Each Drug Intervention Group.

Group	24-h urine protein (mg)	ANA (pg/mL)	dsDNA (pg/mL)
CTX	1.12 ± 1.23	21.44 ± 0.45###	191.63 ± 8.49###
LN	2.13 ± 1.46*	23.99 ± 0.61***	249.48 ± 7.69***
C57	0.75 ± 0.08	21.40 ± 0.77	216.03 ± 6.22
F	3.173	28.378	74.420
P	0.078	0.000	0.000

Note: *compared with C57, # compared with the LN group; */#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$.

Treatment with CTX can decrease the accumulation of immune complexes within the kidneys

In contrast to the LN group of mice, the CTX group showed significant increases in the growth of glomerular cells and also demonstrated vacuolar degeneration in the renal tubular epithelial cells located within the kidney tissue. In contrast, there were no significant alterations observed in segmental lesions, interstitial fibrosis, inflammatory cell infiltration, or vascular lesions (refer to Figure 4). Additionally, the overall immunofluorescence intensity for IgA, IgG, IgM, C1q, and C3 was diminished (see Figure 5).

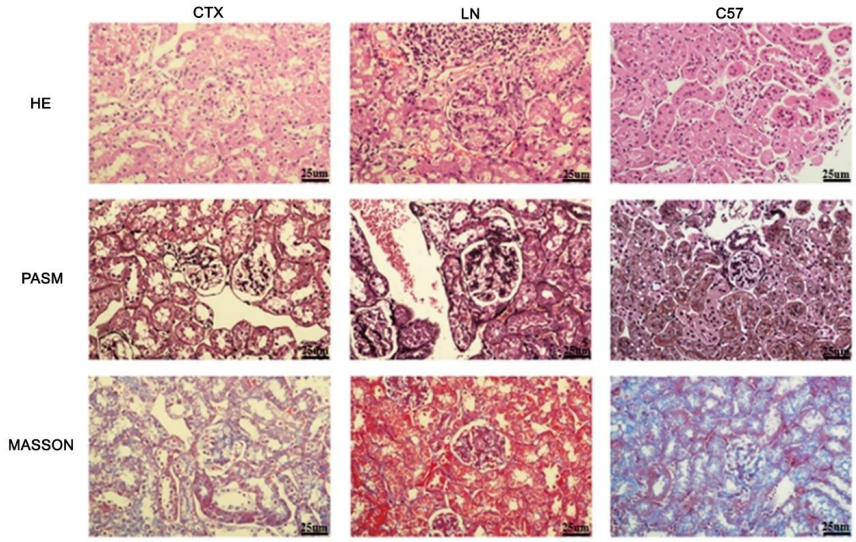

Figure 4. Renal pathology of mice in each group with drug intervention (40 X).

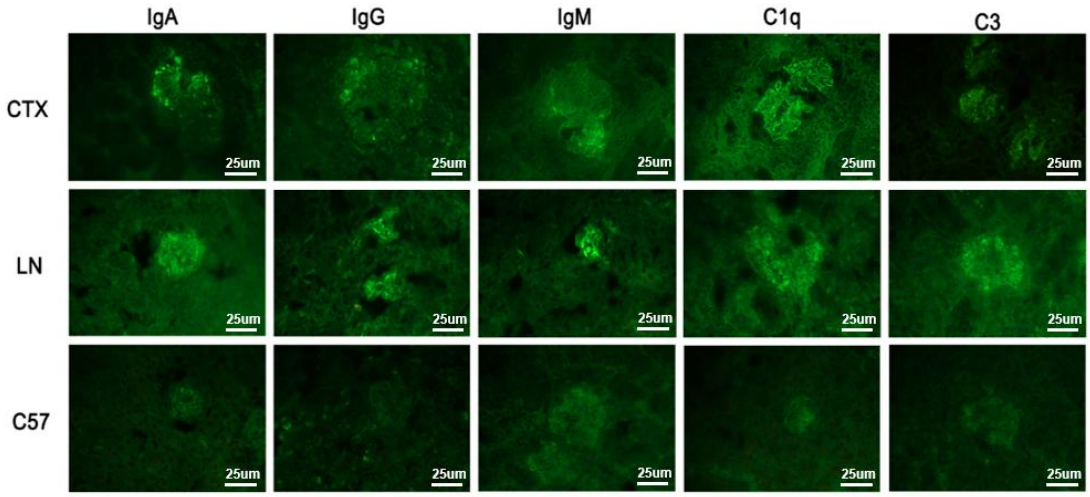

Figure 5. Renal immunofluorescence of mice in each group after drug intervention (40 X).

The administration of CTX treatment leads to a reduction in the expression levels of Gsk 3β and NF-κB p65 within renal tissues

In comparison to the LN group, mice in the CTX group demonstrated a notable decline in both gene and protein expression levels of Gsk 3β and NF-κB p65 in the kidney tissues ($p < 0.01$). Statistically significant differences were noted across all experimental groups ($p < 0.001$, Figure 6a-d).

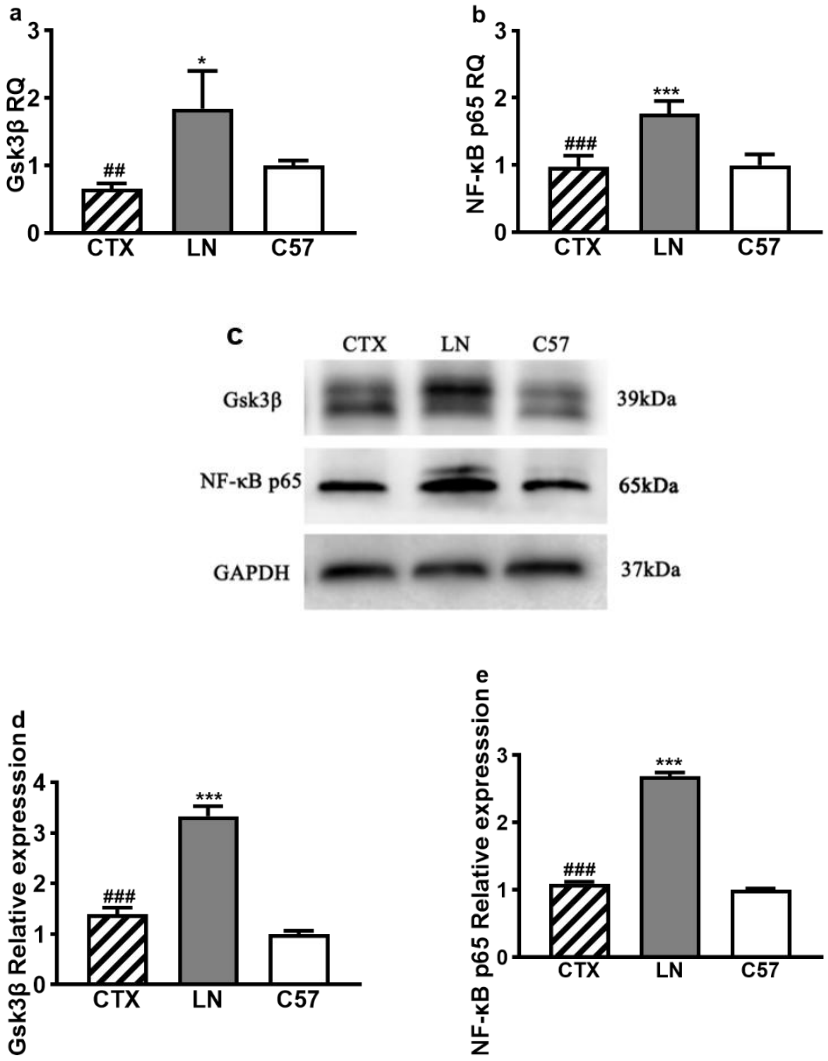

Figure 6. *Compared with the C57 group, #compared with the LN group; */#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$.

Immunostaining for Gsk 3β and NF-κB p65 in mice from the C57 cohort was primarily localized within the membranes and cytoplasm of renal tubular epithelial cells. In contrast, mice from the LN cohort exhibited staining not only in the cytoplasm and membranes of the renal tubular epithelial cells but also within the glomeruli and renal interstitial areas. The degree of intensity of this stain was significantly more than that of the C57 group which differs significantly from each other ($p < 0.05$). The most intense stain is in the glomeruli. The CTX group mice had staining mainly in renal tubular epithelial cells' cytoplasm and membranes. The glomerulus staining was lighter and significantly less than in the LN group. The results were significantly different ($p < 0.05$,

Figures 7-8).

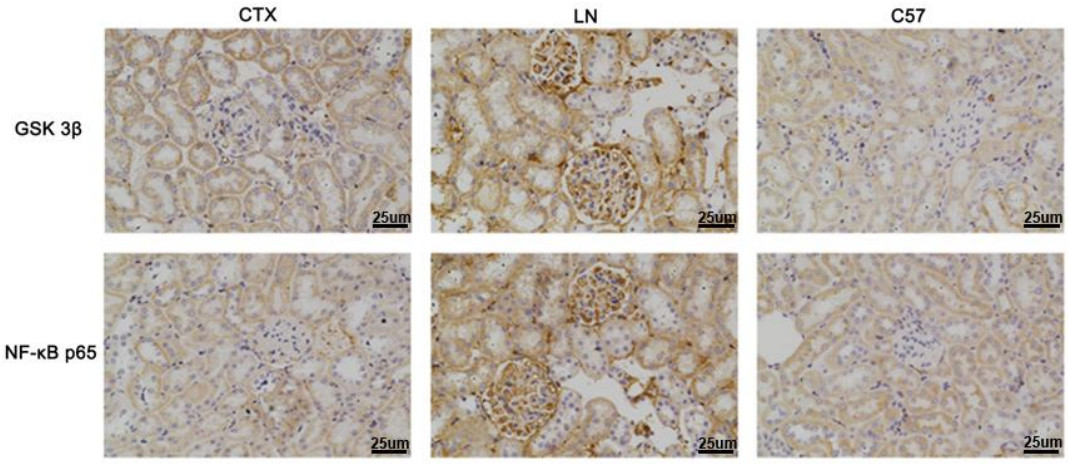

Figure 7. Protein expression of signaling proteins Gsk 3β and NF-κB p65 in different regions of the kidney in mice from various drug intervention groups (40 X).

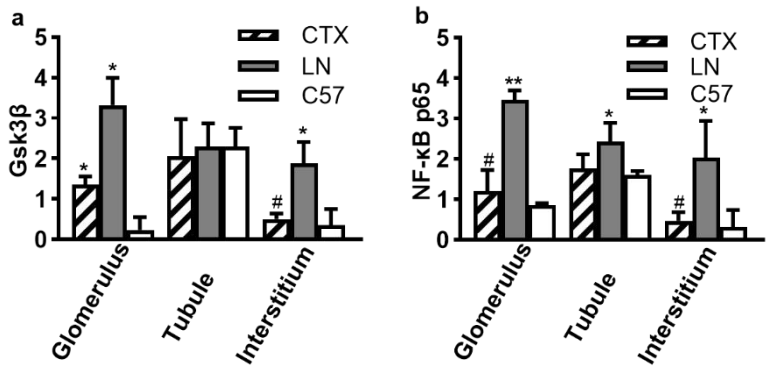

Figure 8. Protein expression levels of signaling proteins Gsk 3β and NF-κB p65 in different regions of the kidney in mice from various drug intervention groups. *Compared with C57, #compared with the LN group; */#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$.

DISCUSSION

During the treatment of LN, despite having made great advances after experimentation, patients with SLE suffer from an overall morbidity and mortality risk. Moreover, research on its disease-causing process hasn't really improved treatment much either. In

addition to routine urine tests, other markers for early LN need to be looked at. If LN patients are identified accurately, it can depose the current model of managing lupus patients from treatment to prevention thereby improving the prognosis.

Gsk 3β is a node for various signaling pathways involved in many physiological processes[10]. It also integrates the host's inflammatory response, functioning as a vital regulator of inflammation and immune reactions. Additionally, it has been associated with the modulation of the transcription factor NF-κB, affecting the inflammatory response through the differential regulation of nuclear levels of NF-κB p65 and the cAMP-response element binding protein (CREB). The p65 subunit of NF-κB promotes gene transcription by interacting with the nuclear coactivator CREB binding protein (CBP). Nevertheless, the availability of CBP in the nucleus is finite, creating a competition for binding between p-CREB and NF-κB p65. Enhancing the interaction between CREB and CBP may inhibit the activity of NF-κB. Studies show that blocking Gsk 3β enhances the interaction between CREB and CBP, whereas it reduces the binding of NF-κB p65 to CBP. Consequently, Gsk 3β influences inflammatory responses via CREB[11], which positions it as a promising therapeutic target for inflammatory disorders. NF-κB serves as a transcription factor for a variety of inflammatory cytokines, and it is involved in immune responses, inflammation, and the proliferation of mesangial cells, making it relevant to various pathological conditions in the kidneys[12]. NF-κB p65 plays a crucial role in the initiation and development of LN by moving into the nucleus, attaching to particular DNA sequences, and triggering target proteins that promote inflammation and immune reactions[13]. Serving as a crucial effector in inflammatory activities, it shows increased activation in areas of inflammation, initiating the inflammatory cascade and promoting the production of cytokines including IL-1, IL-6, and TNF-α[14]. By obstructing the NF-κB pathway via the inhibition of NF-κB p65, it could potentially reduce both the chemotaxis of macrophages and the proliferation of mesangial cells, ultimately decreasing the inflammatory response and alleviating the intensity of renal failure in lupus nephritis models[15,16].

The successful modeling of LN in 12-week-old MRL/lpr mice was confirmed in this study through the assessment of autoantibody levels, including ANA and dsDNA, along with urinary protein and evidence of renal pathological damage. However, the condition did not progressively worsen with increasing age, consistent with previous literature reports[17]. The LN mice demonstrated an increase in glomerular cell numbers, as well as exhibiting segmental lesions and crescent formations. Features also included vacuolar degeneration of epithelial epithelial cells of renal tubules, interstitial fibrosis, infiltration by inflammatory cells, and thickening of the vascular walls. The results of immunofluorescence showed that there were granular or lumpy deposits of IgA, IgG, IgM, C1q, and C3 in the renal tissues of the LN group which could be immune complexes. Also, at 16 weeks, the inflammation could be observed by the presence of inflammatory cells surrounding blood vessels. Necrosis was also noted, suggesting multi-system involvement, with evidence of renal function deterioration with significant proteinuria. At 16 weeks, the presence of proliferative immune complex-mediated glomerulonephritis and vasculitis represents the beginning time point for drug intervention experiments[18].

The expression levels of Gsk 3β and NF-κB p65 genes and proteins were significantly up-regulated in renal tissues of 12-28 weeks-old LN mice. The results of the immunohistochemistry demonstrated the presence of Gsk 3β and NF-κB p65 in the renal tissues of these mice, which are located mainly in the cytoplasm and membranes of the renal tubular epithelial cells in the C57 group. On the contrary, these proteins in the LN group were also localized to glomeruli as well as interstitial cytoplasm and membranes, showing significantly greater staining intensity compared to C57 group. The glomeruli showed the strongest staining, suggesting accumulation of Gsk 3β and NF-κB p65 in this area. All in all, these findings suggest that the increased levels of Gsk 3β and NF-κB p65 are associated with the onset and progression of LN. Studies indicate that Gsk 3β inhibition enhances the stability as well as the function of Treg cells[19]. In MRL/lpr mice, TDZD-8 treatment significantly reduces the incidence of severe

proteinuria (≥ 300 mg/dl) and renal inflammation along with a reduction in serum BUN and dsDNA levels. Moreover, the intervention lessened the accumulation of renal IgG and C3 immune complexes, reduced circulating pro-inflammatory cytokines, and alleviated both glomerulonephritis and interstitial inflammatory infiltration[20]. Mice with ABIN1 gene silencing exhibited abnormal activation of NF-κB and developed lupus-like autoimmunity and pathological symptoms similar to human LN[21]. Research data indicate that inhibition of NF-κB signaling can exert a protective effect on glomeruli, thereby alleviating the pathological damage of LN[16]. These results align with our experimental observations. Therefore, focusing on the signaling pathways of Gsk 3β and NF-κB may offer significant therapeutic options for LN.

The reduced and stabilized risk of end-stage renal disease (ESRD) due to LN aligns with the integration of CTX therapy as a standard treatment[22]. As previously referenced[23], treatment with the drug commenced at 16 weeks of age for LN mice, via intraperitoneal injection at 80 mg/kg CTX, on a weekly basis. After 12 weeks of treatment, levels of antinuclear antibody (ANA), double-stranded DNA (dsDNA), pathological damage and lesser staining for IgA, IgG, IgM, C1q and C3 on immunofluorescence were significantly less as compared to the LN group. After CTX treatment, GSK 3β and NF-κB p65 expression is mainly cytoplasmic in renal tubular epithelial cells. Less staining is found in the cytoplasm of glomerular cells. The levels of GSK 3β and NF-κB p65 gene expression and protein were also significantly decreased in kidney tissues. These results suggest that CTX influences the signaling pathways associated with GSK 3β and NF-κB p65, aligning with findings from other studies in the field[24,25]. CTX alleviates joint pain, tenderness, and swelling metrics by lowering the concentrations of programmed death receptor 1, interferon-induced protein 10, Notch1, and NF-κB in the peripheral blood of individuals with systemic lupus erythematosus (SLE), ultimately leading to a reduction in erythrocyte sedimentation rate and demonstrating its therapeutic benefits[26]. In investigations utilizing animal models of systemic inflammatory response syndrome and LN, Gsk 3β inhibitors, specifically TDZD-8 and LiCl, have demonstrated a significant reduction in the release of

pro-inflammatory cytokines, as well as improvement in renal damage associated with acute kidney injury (AKI) and LN[27-29]. Additionally, artemisinin along with its derivatives has proven effective in addressing the imbalance of immune cells and cytokines linked to systemic lupus erythematosus (SLE) by influencing the TLR-7/9 and NF-κB signaling pathways. This results in improved clinical outcomes, such as decreased proteinuria and renal damage in SLE animal models[30,31]. Consequently, it is suggested that CTX might exert its therapeutic effects by lowering the expression levels of both the Gsk 3β gene and NF-κB p65 protein in the kidneys of mice suffering from lupus nephritis, thus potentially serving as a target for therapeutic intervention.

In conclusion, the involvement of both Gsk 3β and NF-κB p65 in LN progression. Serum concentrations of Gsk 3β may serve as a new biomarker in patients with LN to determine the degree of damage. The study on time gradients carried out using MRL/lpr mice revealed the expression of Gsk 3β and NF-κB p65 starting from 12 weeks of age which may provide an insight into one of the molecular mechanisms involved in the onset and progression of LN. In addition, CTX exhibited therapeutic benefits in MRL/lpr mice due to lower concentrations of the signaling proteins Gsk 3β and NF-κB p65 in the renal tissues affected by LN. This means these proteins may serve as drug targets for CTX for the treatment of LN.

CONCLUSION

The upregulated levels of signaling proteins Gsk 3β and NF-κB p65 and their acting genes may aggravate kidney damage in mice with LN. It is proposed that CTX may exert renal protective effects through inhibition of Gsk 3β and NF-κB p65. Further human studies are still required to validate it, but targeting this pathway may offer a novel therapeutic target.

DECLARATIONS

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Authors' contributions

The input of every author was essential in developing and designing the intervention and study.

Prepared materials, collected data, and carried out analyses: L.L, Z.Y, H.Z, Y.C, N.G, L.L, and SZ;

The initial draft of the manuscript was written by SZ, HZ, and LL, while all authors reviewed and evaluated later iterations.

LL and ZY took charge of translating this article, and the figures were created by ZY, YC, NG, and LL.

Every author thoroughly reviewed, read, and approved the final manuscript.

Availability of data and materials

Those who are interested in the datasets used in this study can request them from the author of it under reasonable conditions.

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Conflict of interest

According to researchers, the study was conducted in the absence of any commercial or financial link that may be considered a potential conflict of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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