Beneficiary Effects of Colchicine on Inflammation and Fibrosis in A Mouse Model of Chronic Kidney Disease

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Research Article

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Abstract

Introduction: Low grade inflammation is seen in many chronic illnesses, including chronic kidney disease (CKD). We have recently reported on beneficiary effects of anti-inflammatory treatment in the interleukin (IL)-1 pathway on anemia as well as CKD extent in a mouse model. Colchicine has been shown to have beneficiary effects in several inflammatory conditions through various mechanisms, including inhibition of tubulin polymerization as well as caspase 1 mediated IL1 activation.

Methods: CKD was induced by administering an adenine diet to 8-week-old C57BL/6J mice. Mice were treated with colchicine (Col) (30µg/kg) or saline injections for 3 weeks, generating 4 groups: C, C-Col, CKD and CKD-Col.

Results: Uremic animals had an increase in inflammation indices in blood (neutrophils), liver and kidneys (p-STAT3, IL-6, SOCS-3). Increased kidney tubulin polymerization and caspase 1 in CKD, as well as kidney Mid88 and IRAK4 (downstream of IL1) were inhibited in CKD-Col. Kidney macrophage infiltration (F4/80 and MAC-2), the percentage of fibrotic area and TGFb mRNA levels were lower in CKD-Col Vs CKD.

Conclusions: colchicine improves kidney macrophage infiltration and fibrosis in CKD through inhibition of tubulin polymerization and Caspase 1 activation. Given its reported safety profile for long term anti-inflammatory therapy without increasing infection tendency, it may serve as novel therapeutic approach in CKD.

Introduction

Chronic kidney disease, like other chronic non infectious conditions, is a state of low grade inflammation through activation of the innate immune system [1]. IL-1 is a key pro-inflammatory cytokine of innate immunity, which plays essential roles in acute and chronic inflammation, host defense and acute phase responses, enhancing inflammatory cell infiltration and augmenting adhesion molecule expression [2]. IL-1b production is tightly controlled, including the production of the pro-IL-1b protein (p35), followed by its cleavage to produce active IL-1b (p17), which is then released into the extracellular environment. Pro-IL-1b processing involves the activation of a caspase-1-activating complex, of which the best characterized component is the inflammasome. Upon activation, the inflammasome is formed by a member of the NALP protein family, such as NALP1, NALP2 or NALP3, and the adaptor protein ASC that connects the NALPs with caspase-1 [3]. Inflammasome activating signals include microorganisms as well as danger signals, such as monosodium urate [4] and other toxic substances that could accumulate in uremia [5].

Clinical and experimental data (for example, in patients with rheumatoid arthritis) show that increased inflammation through this pathway is associated with CKD progression [6] and complications, including erythropoietin resistant anemia [7] and cardiovascular events [8].

We have recently described the beneficial effects of IL1 inhibition in a mouse model of CKD for both anemia and CKD severity [9]. However, because of the concern of long term immunosuppression with this

potent agent in terms of infection tendency, the search for safer agents that inhibit innate immunity continues.

Colchicine is a medication derived from the autumn crocus (*Colchicum autumnale*) plant. It has been used since ancient ages for relief of swelling and rheumatic disorders. Colchicine is an alkaloid that has relatively long terminal half-life and high bioavailability [10]. It has been used for years for treatment and prevention of several diseases such as gout, familial Mediterranean fever (FMF) and pericarditis. Recently, its efficacy has also been shown for the secondary prevention of adverse cardiovascular events [11]. Colchicine has several mechanisms of action, such as a disruption of microtubule polarization by interaction with the microtubule sub unit tubulin, inhibition of cell migration, proliferation, cytokines secretion, reactive oxygen species generation and suppression of inflammasome activation [12].

Few data exist about the effects of colchicine in CKD, maybe because of the concerns with its use when glomerular filtration is decreased. Colchicine may have toxic effects above a certain dosage, which is dependent on the clearance of colchicine from the body by the liver and kidneys [13]. Still, a previous animal study has shown beneficiary effects of colchicine in a rat model of hypertensive CKD [14].

The purpose of our study was to investigate the effects of colchicine on CKD severity, as well as its effects of inflammation through different pathways.

Materials And Methods

Animals

This study was approved by the Ben-Gurion University of the Negev Animal Use and Care Committee, protocol number IL-39-07-2018. All protocols comply with the NIH Guidelines and are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). Animals were housed in standard laboratory cages. Food and water were given *ad libitum*. Eight weeks old male C57BL/6 mice (Harlan Laboratories Inc. Rehovot, Israel) were divided into 4 groups (n=6-10 in each group): C, C-col, CKD and CKD-col. CKD was induced by adenine diet [15]. First an 0.3% adenine, 0.9% phosphorus and 75 ppm iron diet was given for 10 days, switched then to a 0.2% adenine diet and unchanged phosphorus and iron, for additional 11 days. Control groups were fed with a control diet (0.3% phosphorus, ~75 ppm iron). All diets were purchased from Envigo Teklad, (Huntingdon, UK). Colchicine (Sigma, Missouri, USA) was diluted in saline to a 10 mg/ml solution and given at a dose of 30 mg/kg intraperitoneally (ip) to C-Col and CKD-Col mice seven days a week, while C and CKD groups were ip injected with saline. Mice were sacrificed after 21 days, using anesthesia with ketamine and xylazine, collecting: blood, liver and kidney. Serum creatinine levels were analyzed using the AU2700 analyzer (Beckman-Coulter, CA, USA).

RNA extraction and real-time PCR

Assays were performed with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) as previously described [16], using the ABI Prism 7300 Sequence Detection System (Applied

Biosystems, Foster City, CA, USA). Primers for quantification (Sigma-Aldrich, Rehovot, Israel) are summarized in Table 1.

Quantitative analysis of free and polymerized tubulin

Analysis of free and polymerized tubulin was done as described by Ishibashi et al [17]. Briefly, 50mg of kidney tissue was homogenized (polytron, Kinematica, Littau, Switzerland) in microtubule stabilizing buffer (50% glycerol, 5% dimethyl sulfoxide, 10 mmol/L sodium phosphate, 0.5 mmol/L MgSO₄, 0.5mmol/L EGTA). The homogenate was centrifuged and the supernatant containing the free tubulin was kept. The pellet was re-suspended in microtubule depolymerizing buffer (0.25 M sucrose, 10 mmol/L sodium phosphate, and 0.5 mmol/L MgSO₄), centrifuged and the supernatant that contains the polymerized tubulin kept at -80°C for further analysis.

Western immunoblot analysis

The following antibodies were used for evaluation of the kidney extracts: β -Tubulin (Santa Cruz Biotechnology Inc, Dallas TX USA); Caspase 1 (Novus Biologicals, CO, USA); STAT3, p-STAT3 (polyclonal, Cell Signaling Technology Inc. Danvers, MA); GAPDH (Proteintech Group, IL , USA) and β -actin (Clone C4, MP Biomedical Solon, OH, USA), as previously described [18].

Kidney histology

Kidney segments were fixed in 4% formalin for 48 hours, then embedded in paraffin and cut. Kidney sections were deparaffinized, rehydrated and stained with Masson's trichrome (Bio-Optica, Milano, Italy). Fibrotic area quantification of Masson's trichrome staining was performed as described by Chen et al. using an *ImageJ* software [19].

Immunohistochemistry staining was performed as previously described [9]. Primary antibodies against F4/80 (BM-8) (diluted 1:30; Santa Cruz biotechnology Inc., Dallas Texas, USA) and Myeloperoxidase (MPO) (diluted 1:50; Abcam, Cambridge, UK) were used for the immunohistochemistry staining. For image processing, *CellSense Entry* software (MATIMOP, Tel Aviv, Israel) was used.

Data Analysis

Nonparametric Mann-Whitney U test was used to determine significant differences. The null hypotheses were rejected at the 5% level. Values along the manuscript are presented as means $\pm$ standard errors. Asterisk indicate a significant difference between the indicated and the control group, where hash marks indicate a significant difference between the indicated and the CKD group.

Results

Colchicine treatment had no effect on food intake or weight gain, which was suppressed in both CKD groups. Polymerized tubulin levels (Fig. 1) were elevated for more than fourfold comparing to control

group in mice that developed CKD (4.74 ± 0.31 vs. 1 ± 0.24 AU respectively). Administration of colchicine ($30 \mu\text{g/kg BW}$) to CKD mice, significantly reduced tubulin polymerization to 3.39 ± 0.28 fold of control ($p < 0.05$).

CKD was induced by the adenine diet, which causes crystal deposition and inflammation around them in tubulointerstitial areas. The crystals are washed out by the tissue fixation, leaving irregular cavities in the tubulointerstitium. Higher levels of tubulointerstitial fibrosis were seen in CKD Vs. control (Fig. 2A, left lane). Kidney fibrotic area percentage was significantly reduced in the colchicine treated CKD compared to CKD group (9.2 ± 1.4 vs. 17.1 ± 2 % respectively, $p < 0.01$, Fig. 2B). Serum creatinine and kidney TGF- β mRNA levels were elevated in CKD groups Vs. control (Fig. 2C-D). Nevertheless, TGF- β levels were significantly reduced in CKDcol mice (treated with colchicine) compared to CKD (Fig. 2D, 1.82 ± 0.23 vs. 2.74 ± 0.26 fold of C respectively, $p < 0.05$). While polymorphonuclear (MPO positive) cells are located in tubular lumen, macrophages (F4/80 positive) are scattered in the tubulointersitium (Fig. 2A). Both markers were decreased in CKDcol Vs CKD.

Caspase1 activation occurs downstream the NLRP3 inflammasome activation [4, 20]. Myd88 adaptor together with IRAK4 kinase are required for priming and rapid activation of NLRP3 inflammasome [21] and also for fibrogenesis in pericytes [22]. Caspase1 protein levels increased for almost threefold in CKD untreated group (2.85 ± 0.3 vs. 1 ± 0.2 fold of C in CKD and control respectively). Colchicine treatment (CKDcol) significantly reduced Caspase1 protein levels (1.31 ± 0.27 fold of C, $p < 0.01$) (Fig. 3A). Kidney IL- 1β levels (Fig. 3B) were markedly increased in both CKD groups compared to control (5.15 ± 0.68 and 5.36 ± 0.89 fold of C for CKD and CKDcol respectively, $p < 0.001$). Myd88 (Fig. 3C) and IRAK4 (Fig. 3D) levels were elevated in CKD untreated group compared to control (1.38 ± 0.08 and 1.49 ± 0.11 respectively, vs. 1 ± 0.04 and 1 ± 0.07 in C respectively, $p < 0.01$). A significant reduction was observed in CKDcol groups in both Myd88 (1.13 ± 0.07) and IRAK4 (1.07 ± 0.06) compared to CKD groups, $p < 0.05$, Fig. 3C-D).

Kidney IL-6 m-RNA levels (Fig. 4A) raised for up to 20 fold in CKD groups compared to controls (16.36 ± 1.91 and 20.16 ± 3.07 vs. 1 ± 0.31 in CKD, CKDcol and control respectively, $p < 0.001$). Phosphorylated (p)-STAT3/STAT3 protein ratio were also significantly elevated in both CKD groups (Fig. 4B, $p < 0.001$).

However, there was no difference in these inflammatory mediators between CKD and CKDcol.

Discussion

In this study we show beneficial effects of colchicine on kidney inflammation, as well as fibrosis, using a mouse model of tubulointerstitial kidney disease. CKD is another condition of persistent low grade inflammation, through activation of the innate immune system. Even though the use of colchicine for several chronic inflammatory conditions has been reported in humans, potential safety issues, given its metabolism by the kidneys, have hampered choosing it as a therapeutic agent for CKD [23]. Still, patients with mild or moderate renal impairment or those actively receiving hemodialysis do not show accumulation of colchicine [24], and some of its toxic side effects may be due to interaction with other medications such as with clarithromycin and cyclosporine [25]. Because of these concerns, we have used

a relatively low colchicine dose (30ug/kg). Other studies have shown a dose response effect on renal fibrosis and apoptosis with doses as high as 100 ug/kg, for example in a model of unilateral ureteral obstruction [26]. Colchicine may exert its anti inflammatory effects (for example superoxide release after exposure to monosodium urate) at lower doses [27], which may be beneficial for patients with decreased kidney function.

Different danger molecules (such as monosodium urate or cholesterol crystals) may activate the NLRP3 inflammasome within cells to produce interleukin 1 β and interleukin 18 - key mediators in the inflammatory cascade that may drive further damage to the inflamed organ, such as arterial blood vessels. Colchicine may inhibit this pathway and accordingly prevent organ damage [12]. In our study we show decreased inflammation as well as decreased renal fibrosis in colchicine treated uremic mice. The inflammatory pattern was different for polymorphonuclears (MPO positive cells), which localized to tubular lumen, in contrast to macrophages (F4/80 positive cells) which were more spread along the tubulointerstitium (Fig. 2). The anti inflammatory pattern induced by colchicine included its known effects on polymorphonuclear recruitment by tubulin polymerization inhibition (Fig. 1). The inhibitory effects of colchicine on polymorphonuclears relate to inhibition of neutrophil-rolling interactions with the inflamed vasculature and occurs through GEF-H1 (a microtubule-associated Rho-GEF)-dependent neutrophil stimulation pathway [28].

However, the more salient effects of colchicine in this study can be seen for its ability to prevent macrophage recruitment. A similar effect was seen in other models of interstitial kidney damage, such as by cyclosporine [29]. These effects on macrophage recruitment and fibrosis were mediated by a decrease in caspase 1, a key molecule in NLRP3 activation, which is the first step for IL1 release. Such effect could be seen by the changes induced by colchicine on Myd88 and IRAK4 expression (Fig. 3), intracellular molecules also activated through IL1 receptor.

No similar effects could be seen for IL6 (usually controlled by IL1) and STAT3 (a key intracellular molecule of IL6 activation) (Fig. 4). Non immune-mediated cells, including endothelial and many other cells are involved in the direct production of IL-6 in response to various stimuli, through toll like receptor 4 and NF κ B [30]. Uremia is a known state of increase in toxic molecules in blood stream, which may include also damage associated molecular patterns (DAMPs). These molecules upregulate TLR4, which directly induces the elevation of IL6 [31]. In this study, the marked increase in both IL6 and phosphorylated STAT3, as well as the discrepancy between colchicine effects on IL1 Vs IL6 pathways suggest that more specific anti IL6 interventions may be beneficial in CKD.

In conclusion, colchicine improves kidney macrophage infiltration and fibrosis in CKD through inhibition of tubulin polymerization and Caspase 1 activation. Given its reported safety profile for long term anti-inflammatory therapy without increasing infection tendency, it may serve as novel therapeutic approach in CKD.

Declarations

Acknowledgement

Part of the study results were presented at the Annual meeting of the American Society of Nephrology, 2019.

Statement of Ethics

Study approval statement: Ben-Gurion University of the Negev Animal Use and Care Committee, protocol number IL-39-07-2018. All protocols comply with the NIH Guidelines

Conflict of Interest Statement

The authors declare no conflict of interests. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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Author Contributions

Contributions: D.L. and YS conceived the study design, analyzed the experiment data. YS supervised the laboratory experiments. NS and EA performed the experiments and critically reviewed the manuscript. AT reviewed and analyzed the histopathologic analyses. DL wrote the manuscript's first draft

Data Availability Statement

Raw data are available upon request.

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Tables

Table 1: Primers used for real-time PCR analysis

Gene	Sense	Antisense
TGF-β	GCAACATGTGGAAGCTCTACCAGA	GACGTCAAAAGACAGCCACTCA
IL1-β	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
MYD-88	CGATTATCTACAGAGCAAGGAATG	ATAGTGATGAACCGCAGGATAC
IRAK-4	GTCATGACCAGCCGAATCGTG	CAGACACTGGTCAGCAGCAGA
IL-6	CTATACCACTTCACAAGTCGG AGG	TGCACAACCTCTTTTCTCATTTCC
β-actin	GGTCTCAAACATGATCTGGG	GGGTCAGAAGAATTCCTATG

Figures

Figure 1

Reduction in tubulin polymerization in colchicine treated CKD mice. Experimental groups include wild type mice on a regular (C) or adenine diet (CKD), colchicine treated mice on regular diet (Ccol) or adenine (CKDcol). Polymerized tubulin levels in experimental groups (upper panel), and representative western blot analysis (lower panel) of polymerized tubulin in groups. Asterisk indicate a significant difference between the indicated and the control group, where hash marks indicate a significant difference between the indicated and the CKD group. Bands in figure were cropped from the same blot as described in S1.

Figure 2

Renal phenotype and fibrosis of CKD mice. Experimental groups include wild type mice on a regular (C) or adenine diet (CKD), colchicine treated mice on regular diet (Ccol) or adenine (CKDcol). Representative kidney section, from left to right: Masson Trichrome staining; immunohistochemical (IHC) staining for neutrophils (Myeloperoxidase—MPO); IHC staining for macrophages (F4/80). Bar = 50 μ m (A). Kidney fibrotic area percentage as determined by the extent of Masson Trichrome staining, analyzed with the ImageJ software (B). Serum creatinine (mg/dL) (C). Kidney TGF- β mRNA levels (D). Asterisk indicate a

significant difference between the indicated and the control group, where hash marks indicate a significant difference between the indicated and the CKD group.

Figure 3

Inflammation markers in control, CKD and colchicine treated groups. Kidney Caspase1 protein levels and representative western blot analysis (lower panel) (A). Kidney m-RNA levels of Myd88 (B). Kidney mRNA levels of IL-1 β (C). Kidney mRNA levels of IRAK4 (D). Results are presented as mean $\pm$ SE. n=10/group. Asterisk indicate a significant difference between the indicated and the control group, where hash marks indicate a significant difference between the indicated and the CKD group. See S2 for unprocessed caspase blot.

Figure 4

IL6 and p-STAT3/STAT3 levels. Kidney IL-6 mRNA levels (A). Kidney p-STAT3/STAT3 ratio protein levels (upper panel) and a representative western blot analysis of p-STAT3 and STAT3 (lower panel) (B). Results are presented as mean±SE. n=8-10. Asterisk indicate a significant difference between the indicated and the control group. Bands in figure 4b were cropped from the same blot as described in S3.

Supplementary Files

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