Blood Indoxyl Sulfate and TGF-β1 Protein and mRNA Levels in Chronic Kidney Disease: Updated Insights

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Abstract- Chronic kidney disease (CKD) is characterized by systemic inflammation and the accumulation of uremic toxins such as indoxyl sulfate (IS), which induces fibrogenic signaling via transforming growth factor-beta 1 (TGF-β1). Forty CKD patients and ten healthy controls were enrolled. IS was measured by HPLC, TGF-β1 protein by ELISA, and TGF-β1 mRNA by RT-qPCR in PBMCs. IS and TGF-β1 mRNA levels increased significantly with CKD stage, particularly in stages 3 and 4. TGF-β1 mRNA correlated with IS levels (R=0.4, $P<0.01$), while TGF-β1 protein levels were associated with platelet count (R=0.817, $P<0.001$). TGF-β1 mRNA in PBMCs may serve as an early biomarker of CKD-related fibrosis. IS is a key uremic toxin influencing fibrogenic gene expression.

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Introduction

Chronic kidney disease (CKD) is a global health burden associated with progressive renal dysfunction and elevated cardiovascular risk (1). As kidney function declines, uremic toxins accumulate in the blood, exacerbating systemic inflammation and fibrosis (2). Among these toxins, indoxyl sulfate (IS), a gut-derived protein-bound solute, has received increasing attention due to its strong pro-inflammatory and pro-fibrotic effects (3-5). IS originates from tryptophan metabolism by intestinal bacteria and is normally excreted by healthy kidneys, but its serum concentration rises markedly in CKD (2,4). Studies by Lin CJ *et al*., and Niwa *et al.,* have demonstrated that IS promotes renal fibrosis by activating oxidative stress, the aryl hydrocarbon receptor (AhR), and NF-κB signaling pathways (2,3). Transforming growth factor-beta 1 (TGF-β1), a central cytokine in fibrotic signaling, is upregulated in the presence of IS and contributes to extracellular matrix deposition and tissue scarring (5,6). The role of peripheral blood mononuclear cells (PBMCs) in fibrogenesis has also emerged, with recent findings suggesting that circulating immune cells may reflect systemic fibrotic activity through changes in gene expression, including TGF-β1 mRNA (7,8). While previous studies have assessed plasma TGF-β1 protein levels, fewer have explored its transcriptional regulation in PBMCs, which may offer greater diagnostic sensitivity given that plasma levels can be confounded by platelet-derived TGF-β1 (9,10). Assessing TGF-β1 mRNA expression in PBMCs may provide insights into systemic fibrogenesis that plasma protein measurements cannot capture (6,11-23). To the best of our knowledge, this is the first study to assess the relationship between serum IS levels and TGF-β1 mRNA expression in PBMCs across progressive stages of CKD (24-36).

Materials and Methods

Study design

An observational study was conducted involving 40 patients with CKD (stages 1-4) and 10 healthy controls.

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Staging followed KDIGO criteria. Exclusion criteria included diabetes, liver disease, cardiovascular disorders, and malignancies (16).

Sample collection

Fasting blood was drawn and processed to isolate plasma, serum, and PBMCs. Samples were stored at -70° C. RNA quality was confirmed via spectrophotometry and gel electrophoresis.

Laboratory assays

IS Measurement: IS was measured via HPLC with internal standards and fluorescence detection (17).

TGF-β1 Protein: TGF-β1 levels were quantified by commercial ELISA (20).

TGF-β1 mRNA: Expression in PBMCs was evaluated by RT-qPCR, normalized to β-actin, and analyzed by the 2^−ΔΔCt method (20,21).

Statistical analysis

The study protocol was submitted to the Ethics Committee of Tehran University of Medical Sciences for review. The research was conducted in accordance with institutional ethical guidelines and the principles of the Declaration of Helsinki. All participants were informed about the objectives of the study and voluntarily provided written informed consent prior to sample collection

Data were analyzed using ANOVA and Pearson's correlation tests. A *P* below 0.05 was considered statistically significant. We acknowledge that formal correction for multiple testing was not applied to correlation analyses, which represents a limitation in our statistical approach. Data are presented as mean±standard error (SE), where SE was calculated as SD/√n (n=10 for each group). The relatively small SE values reflect the precision of our analytical methods and the homogeneous nature of our carefully selected study cohorts, which were defined by strict inclusion and exclusion criteria. For correlation analyses, exact *P* are reported throughout the manuscript to enhance transparency of borderline significant findings.

Results

Laboratory tests result

Five groups of ten individuals each were studied. Each group comprised five men and five women. Fasting blood sugar (FBS), HbA1c, ALT, AST, and CBC results were used to verify the exclusion criteria. There were no significant differences between groups regarding age, sex, uric acid, and mean blood Na+/K+ levels. Demographic data and the mean levels of BUN, sCr, uCr, serum total protein, urine protein, serum albumin, and uric acid measurements were presented in Table 1.

Table 1. Demographic data and the results of routine blood and urine tests (mean±SE)

	C	S1	S2	S3	S4
Gender(men:women)	5:5	5:5	5:5	5:5	5:5
Age (year)	51.3	49.8	56.4	58.5	60.7
BUN (mg/dl)	12.4 ± 1.39	22.8 ± 2	27.8 ± 2.5	37.2 ± 2.6$^{\#}$	45.4 ± 4.3$^{\#\#\#}$
Uric acid (mg/dl)	5.1 ±1.8	5.2 ± 1.8	5.5 ± 2.02	5.3 ± 2.1	5.2 ± 2.3
sCr (mg/dl)	0.84 ± 0.08	1.4 ± 0.12	2.1 ± 0.15$^{\#\#\#}$	2.79 ± 0.3$^{\#\#\#}$	3.45 ± 0.4$^{\#\#\#}$
serum protein (g/dl)	7.3 ± 1.1	7.2 ± 1	7 ± 1.3	6.8 ± 1.2	6.6 ± 0.95
serum albumin (g/dl)	4.5 ± 0.45	4.5 ± 0.4	4.3 ± 0.5	4.5 ± 0.48	4.1 ± 0.34
uCr (g/24h)	1.7 ± 0.02	1.6 ± 0.03	1.4 ± 0.04	1.2 ± 0.07	1.1 ± 0.1
Urine protein (g/24h)	0.0	0.41 ± 0.08	1.22 ± 0.15	2.14 ± 0.18	2.61 ± 0.21

C: Healthy control subjects (n=10); S1-S4: Patients with chronic kidney disease stages 1-4, respectively (n=10 each); BUN: blood urea nitrogen; sCr: serum creatinine; uCr: urine creatinine. Values are expressed as mean±standard error (SE). Statistical comparisons were performed using one-way ANOVA

#: significant differences with control (*P*<0.05)

##: significant differences with control (*P*<0.01)

###: significant differences with control (*P*<0.001)

Serum indoxyl sulfate levels

Mean and SE of serum IS levels(μM/l) of the five studied groups are presented in Table 2. A marked increase in serum IS levels accompanied progression through CKD stages; however, the increase was particularly pronounced in patients with CKD stages 3 and 4 (19- and 62-fold increases, respectively, compared to healthy controls) (1,2).

Table 2. Mean serum IS, plasma TGF-1-1protein levels, and *P* of between-group differences

	C	S1	S2	S3	S4
Indoxyl sulfate (□M/L)	2.5 ± 0.45	4.3 ± 0.62#	15.3 ± 2.5	47.7 ± 4.25##	155.5 ± 26.04###
TGF-1 (ng/mL)	36.25 ± 4.43	36.24 ± 4.67	38.20 ± 5.82	45.82 ± 6.12	47.53 ± 5.56
TGF-1 mRNA*	1	1.61 ± 0.017	1.89 ± 0.017	2.68 ± 0.017	3.27 ± 0.017

C: Healthy control subjects (n=10); S1-S4: Patients with chronic kidney disease stages 1-4, respectively (n=10 each); TGF-β1: transforming growth factor-beta 1; IS: indoxyl sulfate; PBMC: peripheral blood mononuclear cells. Data presented as mean ± standard error, where SE = SD/√n.

*: *: Fold increase compared to mean control value, expressed as mean±SE for each group. SE calculated as SD/n/√where n=10 per group.

#: significant differences between S1 and S3 (P<0.05)

##: significant differences between S3 and healthy control (P<0.01)

###: significant differences between S4 and four other groups (P<0.001)

Plasma TGF-β1 protein levels

Plasma TGF-β1 protein levels were measured using a standard ELISA kit (Quantikine). The detection limit was 4.6 pg/mL. A weak association between disease progression and increased plasma TGF-β1 levels was observed; however, there were no statistical differences between groups in cytokine levels. Mean and SE of TGF-β1 protein levels were presented in Table 2 (9).

TGF-β1 mRNA level in PBMCs

Both TGF-β1 and β-actin mRNA expression levels in PBMCs were measured using a Real-time PCR assay and expressed as the TGF-β1/β-actin ratio. The mean ratios were used to calculate fold changes in TGF-β1 mRNA expression levels between groups. Fold increases of TGF-β1 mRNA levels of groups compared to the mean value of the control group were presented as mean fold±SE in Table 2. As we see, mRNA levels were 2.68- and 3.27-fold higher in Stage 3 and Stage 4 CKD patients, respectively, compared to controls (a 2-fold increase or more against control was considered statistically significant) (20,21).

Correlations and other findings

Bivariate correlation analyses were performed using either all samples or a single group, depending on the data structure. The correlation between serum IS levels and TGF-β1 was weak but significant (P=0.041, R=0.323). However, increases in serum IS were also associated with similar increases in TGF-β1 mRNA in PBMCs (P<0.01, R=0.4). Correlation between serum IS and urine protein was direct and significant (P<0.01, R=0.42). However, within-group correlation analysis of the two variables showed an inverse relationship between them: S1 (P=0.131, R=-0.511), S2 (P=0.007, R=-0.511), S3 (P<0.001, R=-0.930), S4 (P<0.001, R=-0.955) (see Figure 1) (34). Increases in serum IS levels were associated with significant rises in serum creatinine levels (P<0.001, R=0.56). Correlation between TGF-β1 mRNA (within PBMCs) and plasma TGF-β1 was significant (P<0.01, R=0.367) (Figure 2A). In the same fashion, a strong correlation was found between blood platelet count and plasma TGF-β1 level (P<0.001, R=0.817) (Figure 2B). Correlation between plasma TGF-β1 and urine protein levels was not statistically significant (1-3,9,10).

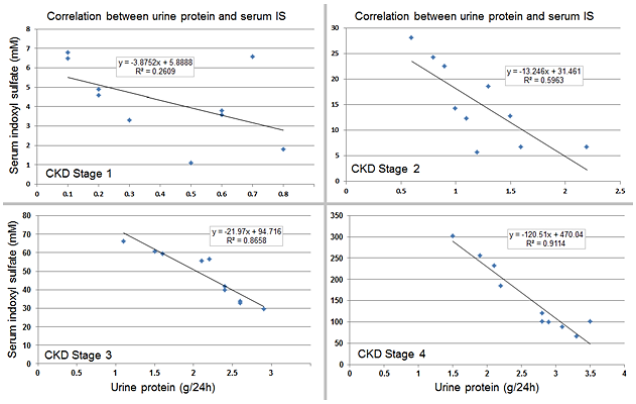

Figure 1. Within-stage correlations between serum indoxyl sulfate (IS) levels and 24-hour urine protein excretion in chronic kidney disease (CKD) patients. Data show inverse correlations within each CKD stage: Stage 1 (S1, P=0.131, R=-0.511), Stage 2 (S2, P=0.007, R=-0.511), Stage 3 (S3, P<0.001, R=-0.930), and Stage 4 (S4, P<0.001, R=-0.955). Each symbol represents an individual patient (n=10 per stage). IS: indoxyl sulfate;

CKD: chronic kidney disease. Pearson correlation analysis was used

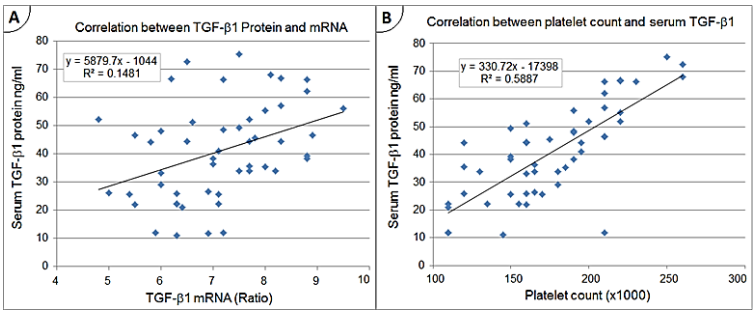

Figure 2. Correlations involving plasma transforming growth factor-beta 1 (TGF-β1) protein levels. (A) Positive correlation between plasma TGF-β1 protein levels and TGF-β1 mRNA expression in peripheral blood mononuclear cells (PBMCs) across all study participants (P<0.01, R=0.367, n=50). (B) Strong positive correlation between plasma TGF-β1 protein levels and blood platelet count across all participants (P<0.001, R=0.817, n=50). Each point represents an individual subject, including both CKD patients and healthy controls. TGF-β1: transforming growth factor-beta 1; PBMCs: peripheral blood mononuclear cells; CKD: chronic kidney disease. Pearson correlation analysis was used

Age has a notable influence on the levels of biochemicals in the body. During the sampling from population, and for minimizing the age interferences on studied variables, we selected samples with a narrower age ranges, however, bivariate correlation analyses of ages with TGF-β1, Plt, TGF-β1 mRNA, sCr, IS, uPr showed direct association of the variables with age, however, correlations between age and IS (P=0.019, R=0.330), sCr (P=0.002, R=0.429) or cytokine mRNA expression in PBMCs (P=0.001, R=0.449) were statistically significant (1,9).

An independent-samples t-test was used to compare variables between the two genders, and only mean creatinine levels were statistically higher in men (mean: 1.03, CI: 0.96-1.1) than in women (mean: 0.77, CI: 0.7-0.84) (P<0.0001). Within-group analyses also showed the differences between the two genders (P<0.05).

Discussion

Our relatively small sample size (n=10 per group) represents a significant limitation that warrants discussion. This sample size provided limited statistical power for detecting smaller effect sizes and prevented detailed subgroup analyses based on demographic or clinical characteristics. With our current sample size, we had approximately 80% power to detect large effect sizes (Cohen's d ≥0.8) but insufficient power for moderate effects. This limitation is particularly relevant for our correlation analyses, where we observed several borderline significant associations that might benefit from larger sample sizes for more robust statistical inference. Our findings reinforce the relationship between IS and fibrogenesis via TGF-β1 expression. Increased mRNA levels in PBMCs align with IS elevation, highlighting transcriptional activation. These findings are in agreement with previous studies by Lin CJ *et al*., (2) and Niwa *et al*., (3), which established IS as a potent inducer of pro-fibrotic responses through oxidative stress and the activation of signaling pathways such as AhR and NF-κB. Guo J *et al*., (9) further demonstrated that plasma TGF-β1 levels may not solely reflect fibrotic activity because they are platelet-derived, suggesting that mRNA quantification is a more specific indicator. The strong correlation between IS and TGF-β1 mRNA, but not with protein levels, supports the concept that IS primarily enhances gene expression rather than systemic secretion. This is particularly relevant given that plasma cytokine levels are influenced by platelet count, as shown by the significant association between TGF-β1 protein and platelet levels (R=0.817, P<0.001) (10). Our study thus adds to a growing body of literature suggesting PBMC-based mRNA analysis may better reflect early transcriptional changes in CKD-related fibrosis. Furthermore, the observed correlation between IS and serum creatinine levels (R=0.56, P<0.001) supports its role as a surrogate marker of renal function deterioration. Overall, these findings highlight the diagnostic potential of mRNA profiling in PBMCs as an accessible, non-invasive biomarker for early fibrotic activity in CKD.

TGF-β1 mRNA in PBMCs serves as an early and sensitive marker of fibrosis in patients with CKD. Unlike protein-based biomarkers, which may be influenced by platelet-derived cytokine release, mRNA expression in PBMCs provides a clearer view of gene-level activation associated with fibrogenic stimuli. Our results

demonstrate that IS not only correlates with conventional kidney damage markers such as creatinine and urine protein, but also significantly enhances TGF-β1 mRNA expression. This reinforces the concept that IS plays a central role in transcriptional regulation of fibrotic mediators. Quantifying TGF-β1 mRNA in PBMCs may offer a non-invasive approach to monitor early-stage fibrotic progression and could help stratify patients based on molecular risk profiles, potentially informing therapeutic decisions.

Limitations

This study has several important limitations. First, its cross-sectional design prevents assessment of causal relationships and temporal dynamics between indoxyl sulfate (IS) levels and TGF-β1 expression. Second, the small sample size (n=10 per group) limited statistical power for detecting moderate effect sizes and precluded subgroup analyses based on medications, dietary habits, comorbid conditions, and demographic factors. Third, we did not account for potential confounders that can influence IS production and TGF-β1 expression, including variations in dietary protein and tryptophan intake, use of medications affecting gut microbiota or renal function, subclinical inflammatory status, and genetic polymorphisms in IS transporters. Lastly, using PBMCs as a surrogate for renal tissue may not fully reflect local fibrotic processes within the kidney. These limitations underscore the need for larger, longitudinal studies with comprehensive covariate assessment to validate and extend our findings. The exclusion of other possible fibrotic markers and unmeasured confounding factors, such as medications, diet, and undetected inflammation, may affect the interpretation of TGF-β1 levels. Longitudinal and mechanistic studies are needed to confirm causal relationships.

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