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RESEARCH ARTICLE

Folic acid induced nephropathy in BALB/C mice

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ABSTRACT

The present study was conducted to learn about the various pathological changes due to folic acid-induced Renal changes in mice, which is a widely used experimental model to study the mechanisms underlying kidney fibrosis. Following high-dose folic acid administration, acute kidney injury is rapidly induced, primarily affecting the tubular epithelium. Clinical pathology, macroscopic, and microscopic changes with Hematoxylin & Eosin and special stains such as Masson Trichrome Stain fibrous tissue proliferation. Along with fibronectin, α-SMA, and Smad expression in the progression of Kidney fibrosis. Taken together, our findings might serve as a rationale for developing prospective innovative renal fibrosis. This present study's finding serves as a pathological change with the marker identification, affecting Renal disease progression.

Keywords: Acute kidney injury, chronic kidney disease, folic acid, nephropathy, renal fibrosis

INTRODUCTION

The kidneys perform a crucial homeostatic function in the maintenance of bodily fluid composition and the elimination of waste materials[1]. Kidney failure is a global public health issue with rising frequency and severity, significant expenditures, and poor results[2]. Kidney disease indicates that your kidneys have been damaged and cannot filter blood as effectively as they should. Clinically, Kidney disease is classified into two types: acute kidney injury (AKI) and chronic kidney disease (CKD), which are closely linked[3-5]. These diseases can be caused by various reasons, including ischemia, sepsis, drug toxicity, overdose, heavy metal exposure, and diabetes[6]. AKI is asymptomatic in mild to moderate cases, and patients are recognized through laboratory tests. On the other hand, patients with severe AKI frequently exhibit symptoms such as lethargy, anorexia, nausea, vomiting, restlessness, confusion, fluid retention, and weight gain. Severe and persistent AKI can result in central nervous system symptoms such as uremic encephalopathy, which includes asterixis, confusion, seizures, and a bleeding tendency due to platelet malfunction and severe anemia. Patients suffering from AKI sometimes have regular urine production, oliguria (less than 400 mL/24 h), or anuria (less than 100 mL/24 h) urine output[7].

Nearly 10% of adults worldwide have been diagnosed with CKD, which is linked to significantly elevated risks for cardiovascular events, development of end-stage renal disease (ESRD), which necessitates dialysis or transplantation, hospital problems, and early mortality[8]. The histological lesion of Kidney function deterioration is Renal fibrosis[9], which is defined by the formation of a matrix as a result of fibroblast trans-differentiation and proliferation[10].

Renal fibrosis is a significant indication of all stages of CKD where there is an excess of ECM build-up and deposition. The pathological fibrillar matrix that forms in the potential space between tubules and peritubular capillaries is characteristic of CKD[11]. Fibrosis and fibrogenesis have been recognized as essential amplifiers of CKD progression rather than a bystander process, according to new findings from animal models of renal disease[12]. Animal models have been employed to solve a wide range of scientific problems from basic research to the development and testing of new vaccines and medications[13]. Scientists have investigated various systems and tested new therapies in animal models before translating their findings to humans due to the striking anatomical and physiological similarities between humans and mammals[14].

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Here we make possible ways to create a FA nephropathy in mice model that reflects human pathophysiology. The main benefit of the FA model is the one-time administration of a high FA concentration, which ensures repeatability[15]. Urinary volume, GFR, and filtration fraction all decrease after FA injection. This is followed by an increase in blood urea nitrogen and creatinine

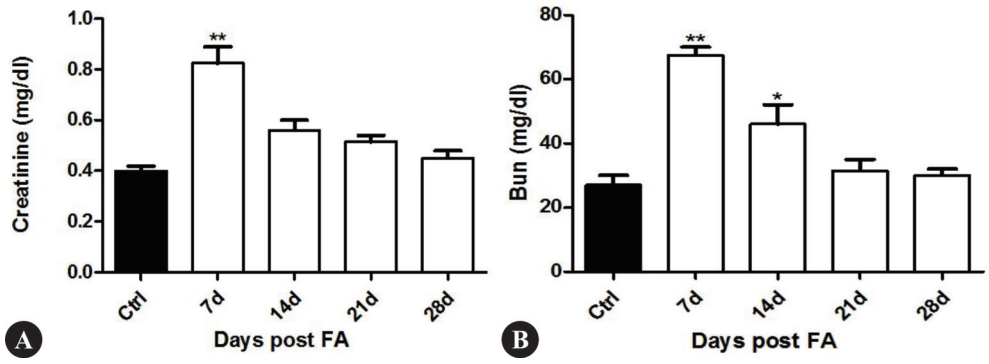


Fig. 1. The time course of the standard renal damage indicators. **A.** Creatinine. **B.** Blood urea nitrogen (BUN). Data represent mean +/- SEM for at least three independent experiments (P<0.05 Dunnet's vs treated).

concentrations[16].

In the present study, we explore various biomarkers that would allow early detection of fibrosis, and also this model has undergone extensive histopathology and renal function analysis. This FA nephropathy mice model, which causes renal fibrosis within 2 weeks of FA injection, should be precious for future renal damage and CKD studies.

MATERIALS AND METHODS

Chemicals and Reagents

Fibronectin (AF5335) and Smad 3 (AF6362) antibodies were purchased from Affinity Biosciences. Smad 2/3 (D7G7) XP® Rabbit mAb #8685, Phospho-Smad 2 (Ser465/467) Smad 3 (Ser423/425) (D27F4) Rabbit mAb #8828 were purchased from Cell Signaling technology. β-actin (sc-4778) were purchased from Santacruz. Anti-mouse (ab6728) and Anti-rabbit (ab6721) were purchased from Abcam (USA). ECL (Enhanced Chemiluminescence), Cytosolic Buffer, Protease inhibitor (PI), Phosphate Kinase inhibitor (PKI) were purchased from Sigma Aldrich.

Animals

Male Balb/c mice, aged 3-5 weeks and weighed 15-30 gm, were obtained from the National Laboratory Animal Facility (NLAF), CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India. Animal husbandry procedures were followed by the recommendations of the institutional animal ethical committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Throughout the investigation, mice were maintained in polypropylene cages at ambient temperature (23±2^{O}C) and relative humidity (55±20%), with a 12-hour light/dark cycle. Access to food and water was unrestricted for all mice. The study was approved by the Institutional Animal Ethics Committee (IAEC) with approval Certificate Reference No. IAEC/2022/43/Renew-0/sl no. 28.

Induction of Renal Fibrosis

Fibrosis was induced by a single injection of folic acid at the dose of 250 mg/kg intraperitoneal (dissolved in Sodium bicarbonate in Mill Q water).

Experimental Design

The total number of animals (n=15) was divided into 2 groups.

Group 1: Normal control mice without FA administration (n=3).

Group 2: Treated mice with FA administration at 250

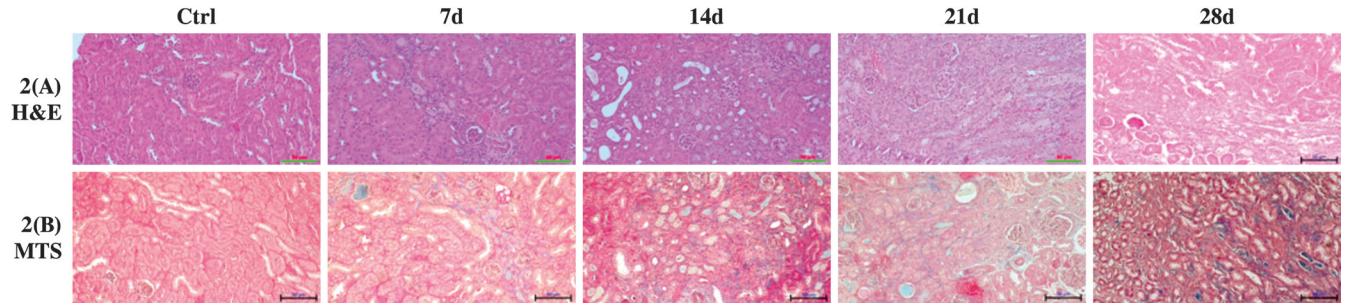


Fig. 2. Evaluation of the renal cortex's damaged region based on histopathology. **A.** Renal damage stage showed with hematoxylin-eosin staining. Normal histology kidney structure is seen in the control group (Ctrl). In contrast, mice treated with FA showed mild interstitial fibrosis and tubular dilation to severe interstitial fibrosis and tubular dilation with localized global and segmental glomerulosclerosis. **B.** Masson trichrome staining with the original magnification at 20x of control (Ctrl), 1 week after FA treatment (7 days), 2 weeks after FA treatment (14 days), 3 weeks after FA treatment (21 days), 4 weeks after FA treatment (28 days). The prominent blue-stained regions in the sections from mice treated with FA Spotted the presence of fibrosis.

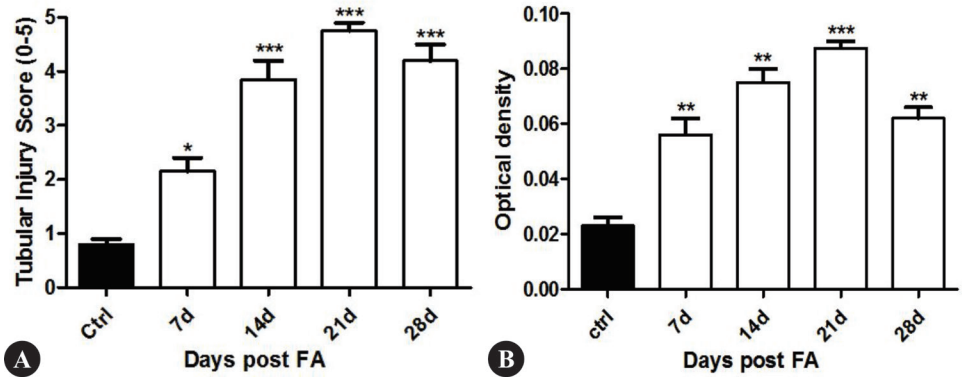


Fig. 3A. Tubular injuries were scored as follows: 0, no tubular injury; 1, <10% tubular damage; 2, 10-25% tubular damage; 3, 26-50% tubular damage; 4, 51-74% tubular damage; and 5, >75% tubular damage. Data represent mean +/- SEM for at least three independent experiments (***P<0.05 Dunnet's vs treated mice). **B.** Quantification of Masson Trichome Positive stained areas. Data represent mean +/- SEM for at least three independent experiments (*P<0.05 Dunnet's vs treated mice).

mg/kg dose.

The study was conducted on days 7, 14, 21 and 28 after folic acid administration (n=3 per group).

Tissue Preparation and Histology

Tissues were fixed in 10% neutral formalin and embedded in paraffin for haematoxylin & Eosin, Masson's trichome, and immunohistochemical staining. Another portion of tissues was stored at -20^{O}C for western blot[17].

Hematoxylin & Eosin (H&E)

H&E staining was performed using a kit from Sigma-Aldrich as per the manufacturer's protocol. The sections were stained with hematoxylin and an eosin stain to observe the structure.

Masson's Trichome

Masson's trichrome staining was executed using a kit from Polysciences (Catalog No. 25088F-100) as per the manufacturer's instructions. The positive area was determined using a bright field microscope (Zeiss).

Immunohistochemistry (IHC)

IHC was performed using an IHC Detection Systems & Kits from Thermo Fisher Scientific (Cat. No. TA-100-DHBH) as per the manufacturer's protocol. The tissue section was incubated in primary antibodies Fibronectin and Smad 3 from Affinity Biosciences overnight at 4^{O}C. Then the sections were incubated in secondary antibody for 3 hrs at room temperature. After washing, the section was incubated with DAB substrate liquid, and the slide was then counter stained with hematoxylin[17].

Western Blot Analysis

Tissues were lysed in cytosolic buffer containing Protease inhibitor (PI) and Phosphate Kinase inhibitor (PKI). The lysates were homogenized (2 Minutes) and then centrifuged (12000 rpm) for 15 minutes at 4^{O}C, and protein estimation was done using the Bradford method. The mixture of protein lysate 40 ug and 2X Laemmli sample buffer was heated for 5 min in a boiling water bath. On SDS-polyacrylamide gel electrophoresis, proteins were resolved using 1X Tris-Glycine-SDS buffer. These resolved or separated proteins were transferred onto a polyvinylidene difluoride membrane (PVDF from Millipore) in a semi-dry transfer unit (BIO-RAD). The membrane was blocked for 1 hour at room temperature in blocking buffer (5% BSA in 0.1% PBST), then incubated with the primary antibody (1:1000) in 2% BSA containing PBST buffer (pH 7.4) overnight at 4^{O}C. After washing with 0.1% PBST buffer (pH 7.4), for 10 min each, the blot was incubated with secondary antibody (1:500) in 2% BSA in PBST buffer (pH 7.4) (dilutions according to manufacturer's instructions) for 2 hrs at room temperature with gentle shaking. After washing with

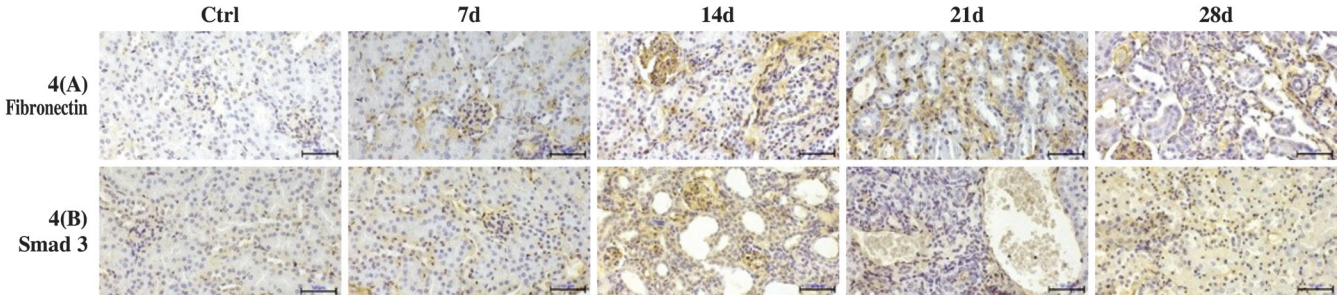


Fig. 4. Chronic renal fibrosis marker proteins: The immunohistochemistry showed the gradual deposition of fibrosis marker proteins. **A.** Fibronectin. **B.** Smad 3 in FA-induced mice from day 7 to day 28 compared with uninduced control group mice.

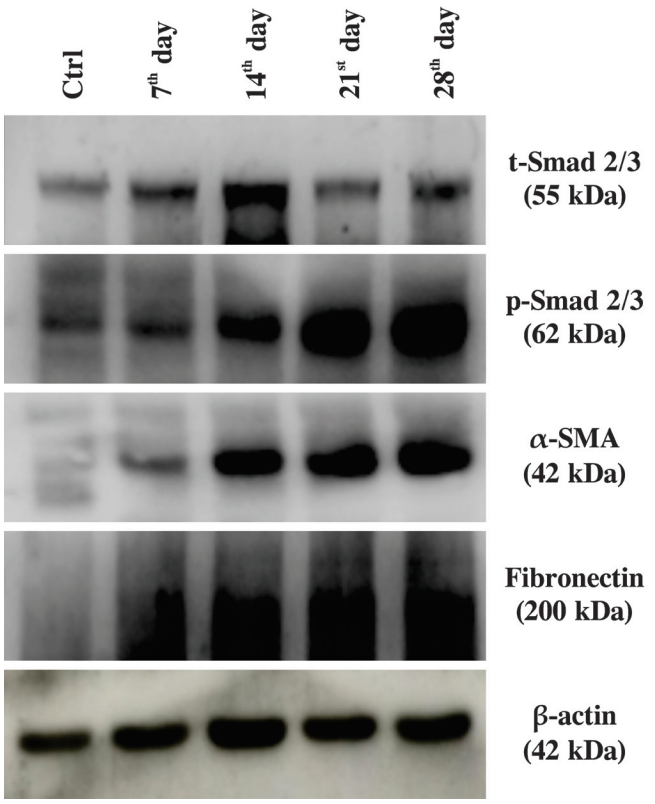


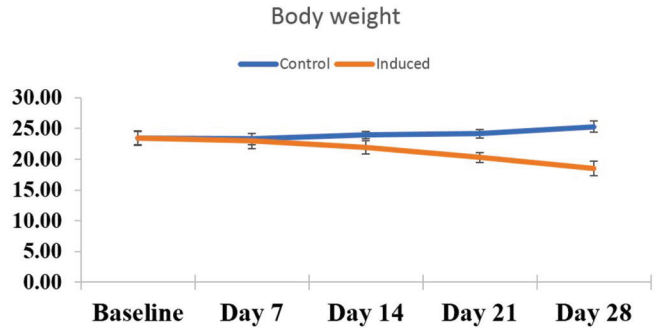
Fig. 5. Expression of renal fibrosis marker proteins in FA-injected mice: The above representative western blot depicts the exponential increase of the early fibrosis marker proteins p-smad 2/3, α-SMA, and fibronectin in the FA-treated group mice (Day 7 to day 28) compared with the control group.

0.1% PBST buffer (pH 7.4), for 10 min each, the protein bands were obtained using Chemidoc Image uant 4000 software.

Statistical Analysis

The data was statistically analysed using the Graph pad prism 5 software package. Each class was examined separately. The unpaired student's test was used to determine statistical significance of numerical data. Differences were deemed significant at $p<0.05$ (*), very significant when $p \leq 0.01$ (**), and extremely significant when $p \leq 0.001$ (***).

RESULTS



Body Weight

We observed that the induced group had less body weight than the control group, and the mice's body weight of the induced group progressively declined over time. We used repeated measures ANOVA to test the bodyweight difference at different time points, and it was found to be significantly different ($p=0.005$). We used the Dunnett test for post hoc comparison and found a significant difference in (induced Day 21) vs (Control Baseline), (Induced Day 28) vs (Control Baseline).

Renal damage Markers

FA significantly affected renal function, as evidenced by elevated plasma levels of BUN and creatinine on day 7 following administration against the control group. A time course analysis showed that the increase in BUN and plasma creatinine levels peaked at 7 days post-FA administration and gradually declined over the subsequent days (Fig. 1A & B).

Renal damage Progression by Histological examination

The renal histopathology of mice induced with FA at weekly intervals was examined using H&E and Masson trichome staining. Minimal interstitial fibrosis, mild tubular dilation, and regenerative tubular cells were observed in the stained section on day 7. On day 14, moderate regenerative tubular cells and tubular dilation were seen in some places with increased interstitial fibrosis and localized global and segmental glomerulosclerosis. Severe tubular dilation and regenerative tubular cells with severe interstitial fibrosis and localized severe glomerulosclerosis were observed on day 21. Mild interstitial fibrosis with minimal regenerative tubules and mild tubular dilation were seen on day 28 (Fig. 2A). The study demonstrated that a single injection of FA increased tubular cell injury compared with the control group.

Masson trichrome staining revealed extensive blue areas on days 14th and 21st, indicating severe fibrosis. Sections with small blue regions on days 7th and 28th showed mild fibrosis (Fig. 2B). These results suggest that FA injection causes progressive renal damage and fibrosis over time, with the most severe damage observed on day 21. These findings may have implications for the management and treatment of FA-induced nephrotoxicity.

As shown in Fig. 3A, there is a progressive increase in tubular injury from day 7 to day 21st and decreased on day 28th. The mean optical densities of the induced group are higher than the Control Group Fig. 3B.

Expression of renal fibrosis marker proteins in FA-induced mice model

The treated group showed positive staining in the nuclei, which was absent in the control group. Fig. 4A & B shows that the staining levels were low in both the control

and 7th day groups. However, there was a significant increase in staining levels in the tubulointerstitial, glomeruli, and extracellular matrix on the 14th, 21st, and 28th days.

FA nephropathy Progression by Western blot

The results showed upregulated expression of p-Smad2/3, α-SMA, and Fibronectin in the FA-induced group, as demonstrated in Fig. 5. Specifically, on day 28th, there was a rise in p-Smad2/3 and α-SMA expression. These findings suggest that Folic acid administration may lead to increased expression of early fibrosis markers, potentially contributing to the development of fibrosis. Our results provide essential insights into the potential mechanisms underlying the observed changes and highlight the need for further investigation into the effects of Folic acid on fibrosis development.

DISCUSSION

It is evident from Fig. 1 that after FA administration, Bun and creatinine levels increase which has also been reported in previous studies[18].

Moreover, the histological alternations associated with renal fibrosis, like tubular dilation, interstitial fibrosis, and glomerulosclerosis, are seen in Fig. 2A, demonstrating the development of FA nephropathy in the FA-induced group (Fig. 3A). The deterioration in Kidney function and progression from AKI to Fibrosis in 2 weeks after FA administration has been reported[19].

A study has already shown that the delivery of FA to mice led to significant tubulointerstitial damage, the establishment and progression of tubulointerstitial fibrosis, and inflammation, as depicted by Masson trichrome staining[20]. This type of outcome was also seen in the present study.

As we know, Smad plays a vital role in renal fibrosis. TGF-β and smad-dependent mechanisms control a wide range of known factors that participate in tissue homeostasis and repair[21-22]. In the present study, IHC showed the deposition of Smad 3 and Fibronectin. The present data demonstrated that FA contributed to the development and progression of FA-induced renal fibrosis.

FA-related AKI is a well-accepted example of nephrotoxic AKI and a widely used model for studies on the processes underlying AKI. Because the FA-induced impairment is restricted to the kidney, extrarenal confounding variables are removed. FA injection causes a local inflammatory response in the kidney that recapitulates upstream signaling of various cellular responses linked to inflammatory conditions critically crucial for tissue remodelling found in many other types of organ damage[23]. The levels of renal damage markers revealed the upregulated expression of p-Smad2/3, α-SMA, and Fibronectin (Fig. 5).

Smad signalling is the primary mechanism by which TGF exerts its biological activity, as well as Smad family members serve specific functions in renal fibrosis. Smad 3 has been reported to have a significant pathogenic role, whereas Smad 7, an inhibitory Smad super family member, counter acts Smad 3's effects[24,25].

CONCLUSION

Our studies have shown that FA elevated the plasma levels of BUN and creatinine on days 7, 14, 21, and 28 following its administration, compared to the control group. Renal histopathology of the treated group showed interstitial fibrosis, regenerative tubular cells, and tubular dilation. In IHC, the mean optical densities of fibronectin and Smad 3 in the treated group were significantly higher than those of normal controls. Based on Western blot analysis, in FA-induced groups, the expression of p-Smad 2/3, Fibronectin, and α-SMA was upregulated in kidneys, indicating the severity of the renal injury. These findings help to understand the pathogenesis of the FA-induced nephropathy mice model and inspire potential therapeutic targets in further studies.

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