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Urinary exosome isolation in fluorosis patients with early symptoms of nephrotoxicity

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Polyureic nephropathy, a kidney disease characterized by excess thirst & frequent urination, are established manifestations of non-skeletal fluorosis. This study aimed to isolate the urinary exosomes from fluorosis patients with early renal impairment. Fluorosis patients having urinary fluoride above 1ppm were recruited as test group (Group-1). The recruited fluorosis patients were sub-divided in two groups i.e., fluorosis patients with normal renal functions (G-1A) and fluorosis paints with renal impairments (G-1B). Healthy individuals having urinary fluoride up to 1ppm were included as control (Group-2). Urinary Exosomes were isolated and correlated with fluoride toxicity associated nephrotoxicity. A significantly (P = 0.002) higher urinary fluoride (G-1A: 2.62 ± 0.94 ppm and G-1B: 3.87±1.6 ppm) in fluorosis patients were observed compared to the normal control (G-2: 0.40±0.19 ppm), (P = 0.004).The serum creatinine levels was significantly increased in G-1B (3.33±0.76 mg/dL) compared to G-1A (1.11±0.89 mg/dL), (P = 0.003) and G-2, (0.64±0.17), (P = 0.005). A significant (P = 0.001) increase in BUN were observed in G-1B, compared to G-1A and control. The urinary protein was also significantly higher in G-1A (P = 0.005) and G-1B (0.001) compared to G-2. The number of exosomes per/ml of urine in G1-B was significantly higher (P = 0.05) than the G-1A and G-2. The number of urinary exosomes in G-1A and G-1B were also significantly higher than G-2 (P = 0.05). Chronic fluoride toxicity may induce renal impairments and increased urinary excretion of exosomes.

Keywords: Atomic Force Microscopy (AFM), Exosomes, Fluorosis, Proteinuria, Renal impairments, Transmission Electron Microscopy (TEM)

Studies have been suggested that chronic fluoride toxicity may induce renal tubular injury[1]. In the persons drinking fluoride contaminated water, kidney function was found to be impaired. On the other hand, skeletal fluorosis due to preexisting kidney disorder is also known[2]. Individual suffering from renal disease may develop skeletal fluorosis even while consuming drinking water containing as low as 1.0 ppm of fluoride. In our previous studies we have been observed various types of fluoride induced ultrastructural changes and increased rate of apoptosis in renal tubular cells of the nephrotic syndrome patients (NSP)[1,3,4]. Also, kidney stone formation is influenced by levels of fluoride in drinking water[3]. Increased free oxygen radical generation and lipid peroxidation have been implicated in the pathogenesis of many diseases and toxic action of a wide range of compounds[5]. Fluoride induced excessive production ROS induces nephrotoxicity[6].

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The available markers of nephropathies (serum creatinine, blood urea nitrogen, proteinuria *etc*.) are late onset markers. In the patients with advanced stages of renal impairment generally urinary and serum fluoride concentrations increases due to compromised GFR and/or restricted water intake and it is difficult to conclude that renal impairment is cause or effect of fluoride toxicity[4]. To find the direct evidence of fluoride associated early kidney injury before increase in serum creatinine & BUN, kidney biopsy is required to see the early changes in different parts of the kidney. Biopsy being an invasive procedure cannot be advised for fluoride exposed subjects. Therefore, identification and validation of non-invasive urinary exosome based early markers well be helpful in early detection and diagnosis of fluoride induced kidney injury.

Exosomes are small vesicular structures released by the cells of the kidneys and express respective cell surface markers. Exosomes carry proteins and nucleic acids, which reflects the physiological state of their respective cells of origin. Accordingly, exosome

vesicles are emerging as a valuable source for disease stage-specific information and indicators of disease progression. However, the potential of urinary exosome based early diagnosis of nephrotoxicity remains unexplored. Proteomic profiling of urine exosomes has identified numbers of proteins originated from kidney cells[7]. Therefore, urinary exosomes may be explored as non-invasive approach to the physiological state of epithelial cells in the kidney. In addition to proteins, urinary exosomes carry a wide range of genetic information molecules like mRNA– encoding proteins native to all nephron regions and, especially small RNA (miRNA) species[8]. Therefore, it is planned to isolate and quantify the urinary exosomes concentration in fluoride associated renal insult at very early stage.

Material and Methods

All the recruited subjects were screened for the fluoride concentration in urine. The recruited subjects were divided in two groups on the basis of urinary fluoride concentration. The subjects having urine fluoride concentration above 1 ppm were considered as test group (Group-1) and the subjects up to 1 ppm of urinary fluoride were considered as control (Group-2). The group-1 was further subdivided in to two sub-groups (G-1A & G-1B) based on kidney function and renal impairment symptoms detailed as follows:

Group-1A: Subjects having urinary fluoride concentration above 1 ppm but having no renal impairment symptoms. Group-1B: Subjects with urinary fluoride concentration above 1 ppm and having early sign of renal impairments (excessive fatigue, feet or ankle swelling and mild micro-albuminuria) Group-2: healthy age & sex matched subjects with normal concentrations of urine fluoride (up to 1 ppm) with no micro-albuminuria, no rise in serum creatinine and blood urea nitrogen (BUN) were considered as control.

Ethical permission was obtained from Institutional Ethics Committee, AIIMS, New Delhi, India, in the year 2019, and the subjects were recruited from fluorosis endemic areas. After obtaining the consent, patients and healthy controls were recruited as per the inclusion criteria.

To the best of our knowledge there are no study found on human subjects and this is the first attempt to assess the urinary exosome in fluoride exposed subjects and associated nephrotoxicity in human subjects. Keeping it in mind to fulfil the primary objectives, 113 subjects were included in the study (G-1A, n=42 and G-1B n=21, and G-2 n=50). A well designed (health indicators) questionnaire pro-forma was used for clinical history recording.

Blood samples were collected and subjected for kidney function tests, following routine protocol. Urine samples were collected in sterile plastic containers and were used for fluoride estimation. Urine was diluted and measured in Total Ionic Strength Adjustment Buffer (TISAB) to maintain constant ionic strength and to remove interferences. Urinary fluoride measurement was done by potentiometric method using fluoride selective ion electrode (Orion, Thermo scientific, USA).

Urine samples (5 mL) were centrifuged at 2000 × g for 30 min at 4°C to remove cells and debris. From the urine supernatant, urinary exosomes rich fractions were isolated by using Total Exosome Isolation (from urine) Kit (Invitrogen, USA, Cat no. 4484452). Briefly, an equal volume of urine and reagent were mixed and incubated at room temperature for 1 hour. After incubation, the samples were centrifuged at 10,000 × g for 1 h at 4°C. After aspiration of the supernatant, a pellet containing urinary exosomes were obtained and used for transmission Electron Microscopy (TEM) for the confirmation of presence of exosomes in urine and semi quantification of urinary exosomes (Fig. 1). Results were expressed as the median or mean/standard errors (SE). The Mann–Whitney *U* test was used to compare two groups (P <0.05).

Results

Fluoride levels in urine

Fluoride is a toxic elements known to cause various health complecations including skeltal fluorosis, dental fluorosis and soft tissue fluorosis. Fluoride induces injuries to many vital organs including kidneys[1,3]. The 1 ppm of urinary fluoride considered to be normal. However, the eeffects of fluoride toxicity vary from person to person depending on the nutritional status and general condition of the patient. Malnourished subjects tend to present with symptoms of fluorosis following exposure to even lesser quantities of fluoride. The impact and severity of fluorosis also depends on the length of exposure to fluoride. The results of this study showed a significantly (P = 0.002) increased concentration of urinary fluoride (G-1A: 2.62 ± 0.94 ppm and G-1B: 3.87±1.6 ppm) in fluorosis patients compared to the normal control (G-2: 0.40±0.19 ppm), (P = 0.004), (Graph-1A).

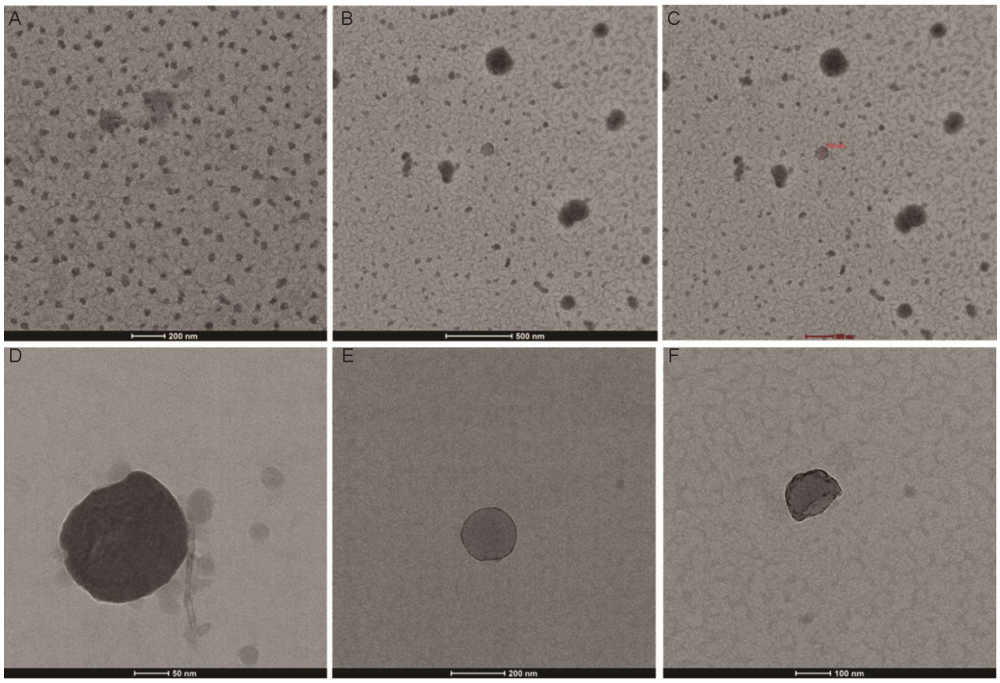
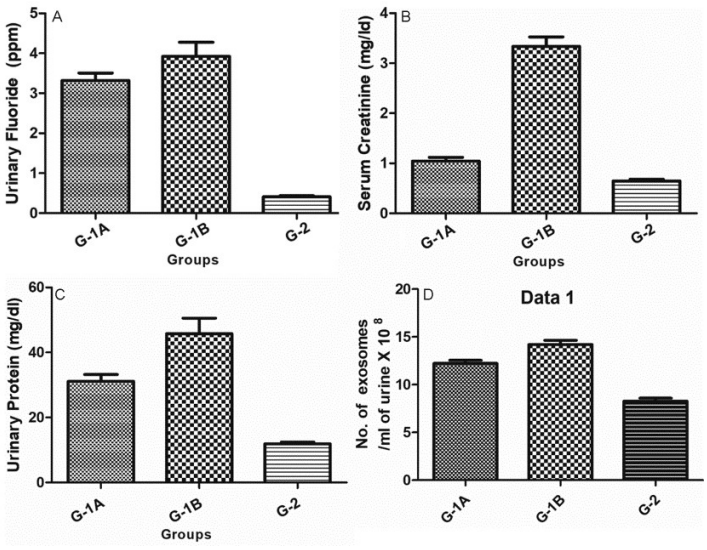


Fig. 1 — Representative transmission electrom micrograph (A-C) showing urinary exosomes at different magnifications; and (D-F) represents individual urinary exosomes



Graph. 1 — Represents urinary fluoride concentration: G-1A: fluorosis patients without renal impairment and G-1B: fluorosis patients with renal impairment symptoms and G-2: control group; Graph2 - represents serum creatinine levels in G-1A (fluorosis patients without renal impairment),G-1B (fluorosis patients with renal impairment symptoms) and G-2 (control group); Graph3- Represents urinary protein excretion in G-1A (fluorosis patients without renal impairment), G-1B (fluorosis patients with renal impairment symptoms) and G-2 (control group); and Graph4-Represents exosomes numbers/ml of urine in G-1A (fluorosis patients without renal impairment), G-1B (fluorosis patients with renal impairment symptoms) and G-2 (control group)

Serum Creatinine

Serum creatinine is the most commonly used marker of kidney functions. The normal level of serum creatinine ranges from 0.7 to 1.2 milligrams per decilitre (mg/dL) in healthy individuals. In the present study it was observed that serum creatinine levels were significantly increased in G-1B (3.33±0.76 mg/dL) compared to G-1A (1.11±0.89 mg/dL), (P = 0.003). The serum creatinine was not significantly increased in G-1A compared to G-2 (control), (0.64±0.17), (P = 0.18), (Graph-1B).

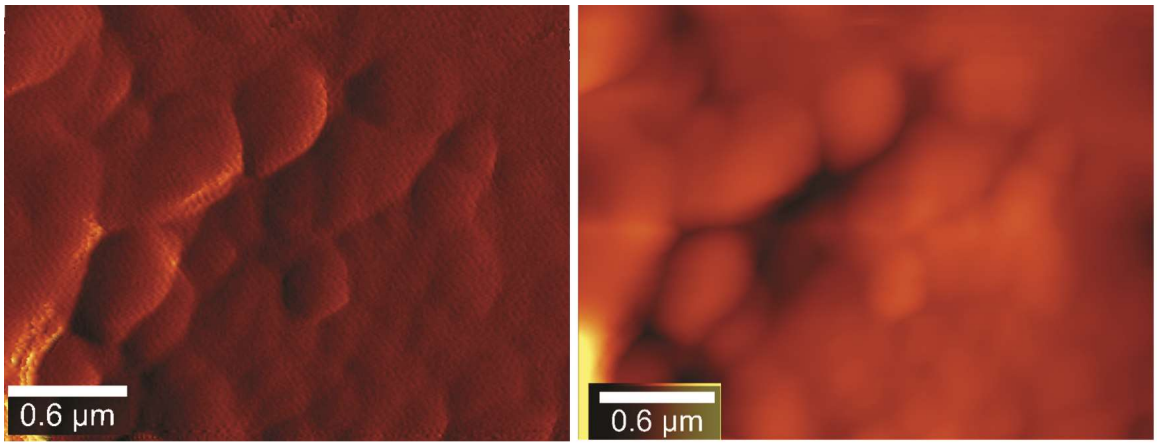


Fig. 2 — Representative Atomic Force Microscope (AFM) micrograph showing urinary exosomes at different phases

Blood Urea Nitrogen (BUN)

Blood urea nitrogen test quantify the amount of urea nitrogen present in the blood. The normal BUN level varies between 7 and 21 milligrams per decilitre (mg/dL). The main cause of an increase in BUN is decrease in glomerular filtration rate (GFR) due to renal impairment. In the present study a significant (P = 0.001) increase in BUN were observed in fluorosis patients having renal impairment symptoms (G-1B) compared to G-1A and control. The BUN levels were mildly increased in fluorosis patients without renal impairment symptoms (G-1A) compared to G-2 but was statistically not significant (P = 0.08), (Graph-1C).

Urinary protein expression

Excessive excretion of protein in urine is indicator of renal impairment. In healthy persons urine contains minute quantity of protein. In the present study an increased quantity of protein excretion was observed in fluorosis patients. The urinary protein was significantly high in G-1A (P = 0.005) and G-1B (0.001) compared to G-2 (control).

Isolation of urine exosomes and TEM analysis

Pellet containing urinary exosomes were subjected to transmission Electron Microscopy (TEM) for the confirmation of presence of exosomes in urine (Fig. 1), and number of urinary exosomes in per field were counted. The result of the TEM analysis showed that the number of exosomes per/ml of urine in fluorosis patients having urinary fluoride in G1B were significantly higher (P = 0.05) than the G-1A and G-2. The number of urinary exosomes in G-1A and G-1B were significantly higher, compared to G-2 (P = 0.05) (Fig. 1 & Graph-D).

The urinary exosomes were also analyzed by Atomic Force Microscopy (AFM) without giving any TEM processing insult. A new sample processing methodology was developed and first time demonstrated in this study. The intact urinary exosomes were visualized by AFM and found that sample processing for transmission electron microscopy doesn't affect urinary exosomes morphology (Fig. 2).

Discussion

Fluorosis is endemic in many countries including India. Many states of India are endemic for fluorosis due to high fluoride concentration in underground water[13]. The fluorosis endemic areas of Sri Lanka, Iran, Poland, China, India etc. have shown very high incidence of chronic kidney disease. Fluoride is known to induce oxidative stress and lipid peroxidation which causes injury to the cells of glomeruli and tubule. Mitochondriopathy, ROS production and localized inflammation contribute to the progress of renal damage[1,3,9,15]. Early renal injury cannot be detected due to unavailability of markers which will represent kidney injury before micro-albuminuria. In the condition non-invasive early detection fluoride induced renal injury by urinary exosomes will be of great benefit. Exosomes carry "cargo" which reflects the physiological state of their respective cells of origin[7]. Exosomes are emerging as a valuable source for disease stage-specific information and as fingerprints of disease progression, yet the full potential of urinary exosomes based early diagnosis in nephrotoxicity remains unexplored.

The analysis of urinary exosomes has proven to be an attractive, non-invasive approach to assess the physiological and pathological status of renal cells of distinct origin. Thus, urinary exosomes will offer a

novel approach to assess the distinct nephron segment-specific molecules and detect changes induced by various renal disease processes[10].

The urinary exosomes bound microRNA-26a correlates with renal function and degree of general fibrosis and promise a feature urinary marker of renal fibrosis. In addition to it, Wilm's tumor-1 (WT-1) protein expression in exosomes appears to increase with the decline of the renal function[8]. Urinary exosomes with WT-1, derived from the podocytes, may thus qualify as a simple marker of podocytes injury[11,12,14,15].

BUN, serum creatinine and urinary albumin are universally used as renal function markers. However, it is difficult to estimate specific injured parts of the kidney. Therefore, isolation, quantification and validation of nephron segment specific urinary exosomes-based markers of nephrotoxicity will be of great clinical relevance. The present finding showed that fluoride associated renal injuries increases the excretion of exosomes in urine. The detailed profiling of exosomal proteins and miRNA will pave the way in identification and validation of non-invasive urinary exosome based diagnostic markers of kidney injury.

Conclusion

Patients chronically exposed to high concentration of fluoride may develop renal impairmeents. The urinary excretion of exosomes increases in chronic fluorosis toxicity. The urinary exosome bound various proteins and miRNAs may be explored in further studies.

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Conflict of interest

All authors declare no conflict of interest.

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