

RESEARCH ARTICLE

Environmental Toxicology

Single and combined effect of fluoride and hardness of drinking water on nephrotoxicity: *in-vivo* study using Wistar rats as an animal model

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Abstract: Drinking water in areas with a high prevalence of chronic kidney disease of unknown aetiology (CKDu) in Sri Lanka, is known to have high concentrations of fluoride and hardness. The present study evaluated the individual and combined effects of water hardness and fluoride on potential nephrotoxicity, using Wistar rats as an animal model. Thirty-five Wistar rats were randomly assigned into five groups (n=7). Test groups F, H, RL, and RH were given de-ionized water containing 1.5 mg/L fluoride, 200 mg/L hardness, 1.5:200 mg/L fluoride: hardness, and 5:800 mg/L fluoride: hardness respectively, while control group C was given de-ionized water. Body weight and daily water consumption were measured. Serum creatinine, urine creatinine, and urinary biomarker KIM-1 were analyzed. Histopathological changes in the kidneys were observed. There were no significant differences in body weights ($p>0.05$) while daily water consumption was reduced significantly in the test groups RL and RH ($p<0.05$). A significant increment in serum creatinine in the RL and RH groups ($p<0.05$), and a significant reduction in urine creatinine in the F, H, RL and RH groups ($p<0.05$), were recorded compared to the control. However, the highest magnitude of the effect on serum creatinine and urine creatinine was recorded in the RL group. Significant increment in KIM-1 levels were recorded in the RH group ($p<0.05$) while the RH group indicated a more rapid increment from the 28th day. When considering histopathology, renal tubular changes were observed in the test groups. The individual and combined effects of water hardness and fluoride may contribute to the aetiology of CKDu in Sri Lanka.

Keywords: CKDu, combined effect, fluoride, hardness, *in-vivo*, nephrotoxic effect.

INTRODUCTION

Chronic kidney disease (CKD) is a significant public health concern. It is the 12th major cause of death and the 17th leading cause of disability worldwide (Veerappan & Abraham, 2013). Though conventional CKD is caused by traditional risk factors such as diabetes, hypertension, and high cholesterol, chronic kidney disease of unknown aetiology (CKDu) appears to be a new form of CKD as it is not associated with the conventional risk factors listed above (Gifford *et al.*, 2017). The prevalence of CKDu has been on the rise in Sri Lanka for the past two decades. First reported in the North Central Province of Sri Lanka, the disease later spread to North Western, Uva, Eastern, and Northern Provinces (Athuraliya *et al.*, 2011; Wanigasuriya, 2014). CKDu remains concentrated in the country's dry zone regions (Wanigasuriya, 2014).

Considering the discrete geographical locations, risk factors for CKDu, and clustering of cases, CKDu was postulated to be an environmental disease (Wanigasuriya *et al.*, 2007). It was hypothesized that CKDu in Sri Lanka is partially attributed to groundwater, which is the primary source of drinking water in CKDu endemic districts. Drinking water in areas with a high prevalence of CKDu is known to have a high concentration of fluoride and hardness. Further, heavy metals, cyanotoxins, pesticides, and fertilizers are under investigation as potential risk factors.

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The fluoride anion has the smallest radius and atomic weight of all the halogens, which facilitates entry into cells, easily. Fluorine toxicity may be exacerbated in soft tissues since they lack the buffer factors found in bones, where apatite is considered to be the factor that neutralizes fluoride ions (Birkner *et al.*, 2006). Furthermore, kidneys, which are responsible for efficient fluoride excretion, play an important role in fluoride regulation (Cárdenas-González *et al.*, 2013). The recommended value for the concentration of fluoride in drinking water by the World Health Organization is 1.5 mg/L while, the Sri Lanka Standard Institute (SLSI) guidelines recommend a 1 mg/L value for the concentration of fluoride in drinking water (WHO, 1993; SLS 614: 2013).

Water with a high concentration of calcium and magnesium ions, is generally indicated as hard water. Other dissolved metals, such as aluminum, barium, strontium, iron, zinc, and manganese, can cause hardness in the form of divalent or multivalent cations. Even though hard water has no known adverse health impacts, it could significantly supplement total calcium and magnesium intake (Galan *et al.*, 2002). Health implications of intake of hard water are primarily attributable to its salts, mainly calcium and magnesium. More than 75% of kidney stones are calcium salts, which frequently appear as calcium oxalate and less commonly as calcium phosphate (Sengupta, 2013).

Existing evidences suggest that the combination of fluoride and hardness in ground water may contribute to the aetiology of CKDu in Sri Lanka. This hypothesis is due to the overlap of geographical distribution of fluoride and hardness in ground water and the CKDu prevalent regions in Sri Lanka. The areas with high CKDu prevalence such as Anuradhapura (18.9%) and Polonnaruwa (13.9%), (Kafle *et al.*, 2019) have recorded high concentrations of both fluoride (Anuradhapura >2 mg/L, Polonnaruwa > 1 mg/L) (Chandrajith *et al.*, 2011) and hardness (Anuradhapura 241-300 mg/L, Polonnaruwa 181-240 mg/L) (Indika *et al.*, 2022). The wet zone areas are identified as CKDu non- prevalent areas and they have recorded low concentrations of both fluoride and hardness (Chandrajith *et al.*, 2011). Although, Ampara district has been highlighted as an area with high fluoride concentrations (1.0 -1.5 mg/L), the level of hardness of water as well as the CKDu prevalence was recognized in minute levels (Ranasinghe *et al.*, 2019). Furthermore, Jaffna and Mannar districts have recorded high hardness values while the concentration of fluoride in the ground water (<0.5 mg/L) and the CKDu prevalence was very low (Chandrajith *et al.* 2011; Ranasinghe *et al.*, 2019).

According to the studies so far, the multifactorial effect of water chemistry, emphasizing fluoride and hardness, is considered substantial. Based on the findings mentioned above, the present study seeks to evaluate the *in-vivo* single and combined effects of fluoride and hardness in drinking water utilizing Wistar rats model as an animal model.

MATERIALS AND METHODS

Ethics approval

The Ethics Review Committee of the Institute of Biology, Sri Lanka (ERCIOBSL 194062019) granted ethical permission for the experimental procedure. The animal experimental procedures were carried out in accordance with their guidelines on housing, husbandary, animal care, and monitoring, as well as the guidelines laid down by European Community Directive 86/609/EEC were followed during the experiment.

The experiments employed *Rattus norvegicus*, Wistar strain (origin: CLEA, Japan) bred at the Animal Centre in Medical Research Institute (MRI), Sri Lanka, under microbiologically controlled conditions. Thirty-five (35) male Wistar rats, aged eight weeks and weighing 150-250 g, were recruited for the study. The rats were allowed to acclimatize to the animal house settings for 8-10 days. The Animal House conditions maintained during the study period were; 12:12 hour light-dark cycle, temperature of 25-26°C, and humidity of 40–70%. Furthermore, standard rat cages of 1500 cm² × 24 cm were used, with autoclaved wooden shavings as bedding material and two animals were housed in each rat cage. A standard pellet diet for the Wistar rats was prepared with locally available food ingredients. Considering the CKDu prevalence among the human population, recorded evidence showed high incidents in males (Ruwanpathirana *et al.*, 2019; Pett *et al.*, 2022). Therefore, considering previous literature, male animals were recruited for the current experiment (Ruwanpathirana *et al.*, 2019; Pett *et al.*, 2022).

Chemicals

Sodium fluoride (NaF) with $\geq 99\%$ purity, Calcium carbonate (CaCO_3) with $\geq 99\%$ purity, and Magnesium chloride (MgCl_2) with $\geq 98\%$ purity, were purchased from Sigma Aldrich, USA and these were dissolved in de-ionized water to prepare the fluoride and hard water samples which were given to rats.

Design of the experiment

The experiment utilized de-ionized water and laboratory-made water samples to evaluate the individual and combined effects of water hardness and fluoride on the kidneys of rats. A water sample with a fluoride concentration of 1.5 mg/L was used to test the effect of fluoride, while a water sample with a hardness concentration of 200 mg/L was used to test the effect of water hardness. Two mixtures having fluoride: hardness concentration combinations of 1.5:200 and 5.0:800 mg/L, were used to detect the combined effect of fluoride and hardness. The ratio between Ca^{2+} to Mg^{2+} used to prepare drinking water with 200 mg/L and 800 mg/L total hardness was 4:1, according to the recommendations of the Organization for Economic Cooperation and Development (OECD, 1992) and the ratio was associated with the Ca^{2+} and Mg^{2+} content in drinking water sources in CKDu prevalent areas. The treated concentrations were selected considering the average concentration distributions of fluoride and hardness in CKDu prevalent areas as well as the recommended concentration levels specified by WHO and SLSI for drinking water.

After ten days of acclimatization, 35 rats were divided into five groups, each with seven rats (C, F, H, RL, and RH). Before treatment, the weights of the rats were recorded and used as baseline body weights. The control group (C) received de-ionized water daily. Group F received 1.5 mg/L of NaF dissolved in de-ionized water daily, while group H received 200 mg/L of CaCO_3 and MgCl_2 dissolved in de-ionized water daily. Group RL received NaF, CaCO_3 , and MgCl_2 dissolved in de-ionized water at fluoride: hardness concentrations of 1.5:200 mg/L daily, whereas group RH received NaF, CaCO_3 , and MgCl_2 dissolved in de-ionized water at fluoride: hardness concentrations of 5.0:800 mg/L daily. Water samples were given to all of the groups for a duration of 90 days and each individual rat was allowed access to water from the respective water samples *ad libitum*.

Sample size calculation

Sample size per group = $2 \text{SD}^2 (Z_{\alpha/2} + Z_{\beta})^2 / d^2$ (Charan & Kantharia, 2013).

SD - Standard deviation from previous study by Thammitiyagodage *et al.*, 2017

$Z_{\alpha/2} = Z_{0.05/2} = Z_{0.025} = 1.96$ (From Z table) at type 1 error of 5%

$Z_{\beta} = Z_{0.20} = 0.842$ (From Z table) at 80% power

d = effect size = difference between mean value

Hence,

$$\begin{aligned} \text{Sample size} &= 2 \text{SD}^2 (1.96 + 0.842)^2 / d^2 \\ &= 2 (10.37)^2 (1.96 + 0.842)^2 / 17^2 \\ &= 5.84 \sim 6 \end{aligned}$$

Expected attrition or death of animals: 10%

For 10% attrition ($6/0.9 = 6.7$) ~ 7

Bodyweight gain, absolute weight, and relative organ weights

During the experiment, the body weights of each group of rats were recorded twice a week. The final body weight of each rat was measured on the day of sacrifice, and the bodyweight gain was calculated by subtracting the final body weight from the initial body weight. The absolute and relative organ weights were determined.

$$\text{Relative organ weight} = \frac{\text{Fresh organ weight (g)}}{\text{Body weight (g)}} \times 100$$

Sample collection

Blood: Blood was drawn from the lateral tail vein (Lee & Goosens, 2015) of rats that had been anesthetized using CO₂ anaesthesia and placed in a rat holder. At days 0, 7, 14, 28, 42, 60, and 90, blood samples were drawn with 27 gauge needles and 1 mL syringers. After performing cardiac punctures on the animals, the final blood samples were taken. After centrifugation at 5000 rpm for 5 min, the serum was separated from the blood and kept at -20 °C until it was used for serum biochemistry analysis.

Urine: Each rat was placed in a metabolic cage for 24 h to collect urine at days 0, 7, 14, 28, 42, 60, and 90 which were stored at -80 °C until the analysis was completed.

Urine analysis and serum biochemistry

The levels of creatinine in the urine of each rat were measured using commercially available creatinine - Jaffe multifunctional reagents (DiaSys Diagnostic Systems, Germany). KIM-1 was tested as a urinary biomarker to diagnose acute kidney damage using the urine samples collected. The expression of KIM-1 was evaluated using an ELISA kit from Elab Science, USA (Catalog Number: E-EL-R3019). Quality assurance and quality control measures were followed to assure the precision of the serum biochemistry and urine tests. Sampling procedure, sampling plan, transportation, storage, and sample analysis were well planned prior to the study and quality control samples were maintained. Furthermore, calibration curves, and check lists were prepared and measurements on two different instruments were obtained for accurate readings.

Histopathology

After 90 days, the rats were euthanized using CO₂ anaesthesia and final blood samples were taken after sacrificing the animals by performing cardiac punctures. Each rat was dissected, the kidneys removed, and the removed kidneys were weighed on a digital scale. Each kidney was bi-valved, and both cut surfaces were examined for colour changes, necrosis, or fibrosis. Kidney tissues were fixed in 10% formalin for 24-48 h and then dehydrated in a series of ethanol concentrations and cleared by using xylene. The tissues were then paraffin-embedded. Hematoxylin and Eosin stains were used to stain kidney tissues and the slides were examined under a light microscope. The significant levels of renal histological lesions were graded using scoring classification defined by Toblli *et al.*, (2019) and the lesions were classified as mild, moderate, and severe. All histopathological studies were conducted at the Department of Pathology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka.

The animals were disposed of according to the guidelines of the Animal House of the Faculty of Medical Sciences, University of Sri Jayewardenepura, and were buried at a designated location.

Statistical analysis

The data are presented as means and standard deviations. One-way analysis of variance (ANOVA) was used to detect the levels of statistical significance between groups with a $p < 0.05$ limit. Furthermore, Tukey's test was used to compare means of different treatment groups. The Minitab 14 statistical software package for Windows was used for statistical calculations.

RESULTS AND DISCUSSION

The present study explored the individual and combined effects of fluoride and hardness as a novel hypothesis on CKDu aetiology. There is a geographic correlation of high CKDu prevalence regions and drinking water with high concentrations of both fluoride and hardness suggesting a combined effect of fluoride and hardness on nephrotoxicity (Water Resources Board, Sri Lanka 2016; Dissanayake & Chandrajith, 2017). Chandrajith *et al.*, (2011) argued that the cytotoxicity effect of fluoride appears to be the effect of Na⁺ and Ca²⁺ ratio of the ingested water on the F⁻ metabolism. Acute tubular injury with elevated serum creatinine was observed in rats fed with hard water with high fluoride content, and the changes were minimized by administration of distilled water (Perera *et al.*, 2020).

In this study, the weight gain of the animals, which is a toxicological indicator, was assessed. The body weights of rats in all the groups increased gradually during the experiment, with no significant differences ($p>0.05$) in weight gain between the treated groups compared with the control group (Figure 1). The body weights gradually increased until the 12th week, after which they remained at the same level.

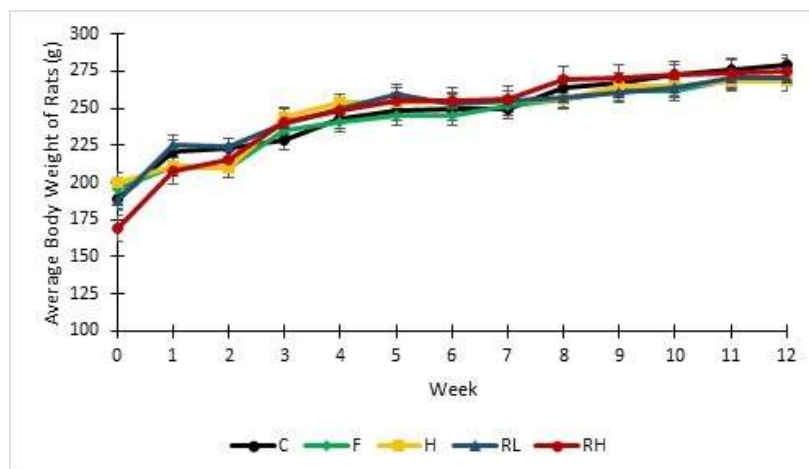


Figure 1: Variation in body weight of rats treated with de-ionized water and test water ad-libitum for 90 days. Data are presented as mean $\pm$ SD. (N=7). C- control, F- group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L

Previous studies have shown that a reduction in organ weight was associated with chemically- induced organ damage. To assess the effect of fluoride and hardness on the kidneys, the relative organ weights of the kidneys (organ weight to body weight ratio) was measured. Compared to the control group, the absolute and relative weight of both right and left kidneys in the RL and RH groups were marginally higher at the end of the experiment however, it is not statistically significant ($p>0.05$) (Table. 1).

Table 1: Absolute and relative weight of kidneys of rats treated with de-ionized water and test water ad-libitum for 90 days.

	Right kidney		Left kidney	
	Absolute weight	Relative weight	Absolute weight	Relative weight
C	0.68 $\pm$ 0.04	0.24 $\pm$ 0.01	0.68 $\pm$ 0.02	0.24 $\pm$ 0.01
F	0.69 $\pm$ 0.04	0.26 $\pm$ 0.02	0.68 $\pm$ 0.05	0.25 $\pm$ 0.03
H	0.66 $\pm$ 0.06	0.23 $\pm$ 0.01	0.65 $\pm$ 0.04	0.23 $\pm$ 0.01
RL	0.70 $\pm$ 0.02	0.25 $\pm$ 0.01	0.70 $\pm$ 0.03	0.25 $\pm$ 0.01
RH	0.74 $\pm$ 0.03	0.26 $\pm$ 0.01	0.76 $\pm$ 0.02	0.27 $\pm$ 0.01

Data are presented as mean $\pm$ SD. (N=7). (C- control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L)

Toxicological studies done in animals have shown that decrease in weight gain precedes organ damage and the development of other clinical signs (Chapman *et al.*, 2013). In the current study, there was no significant difference in body weight gain between the test groups and the control group, indicating that fluoride and hardness had no effect on weight gain in rats during the 90-day experiment. Previous studies have shown that chemical toxicity was associated with changes in body weight gain and the organ weight in experimental animals (Manage *et al.*, 2009; Lazic *et al.*, 2020). A decrease in kidney weight has been linked to renal toxicity, chronic progressive nephropathy, and tubular hypertrophy (Hard *et al.*, 2013). The results of this study showed a slight increase in the

relative weights of both right and left kidneys in the test groups F, RL, and RH, as well as a slight decrease in the H group, suggesting that there was no significant effect on the kidneys of rats in the treated groups compared to the control group. Furthermore, compared to the control group, the difference in body weight gain, absolute weight, and relative weight of kidneys was not statistically significant ($p>0.05$). Perera *et al.*, (2020) found no significant differences in the relative weight of kidneys in the treated group compared to the control group after treating test water samples collected from Mihintale with high concentrations of hardness and fluoride and normal samples collected from Kandy. A study done by Wasana *et al.*, (2017) using ICR female mice to evaluate the synergistic effects of the heavy metals arsenic, aluminium, hardness, and fluoride in water on kidneys, showed a weight loss in all the treatment groups while a weight gain was observed in the control group.

Rats were routinely examined for behavioral changes such as salivation, cannibalism, lethargy. However, no significant behavioral changes were noticed in any group of rats throughout the experimental period. The Tukey's pairwise comparisons indicate that the differences in the daily water consumption of the rat groups F and H were not significantly different from the control ($p>0.05$) while the RL and RH groups were significantly different ($p<0.05$). According to the results of the present study, water consumption in test groups were lower than the control group and the RL and RH groups indicated a prominent reduction in the daily water consumption during the experiment (Figure 2). According to the findings of the study conducted by Wasana *et al.*, (2017) to examine the synergistic effect of heavy metals, fluoride, and hardness, no significant differences in the volume of water consumed by mice in the test and control groups were observed. However, a study done by Siglin *et al.*, (2000) evaluated the subchronic toxicity of environmental contaminant perchlorate in Sprague-Dawley rats and revealed occasional significant reductions in consumption of water by rats over 90 days.

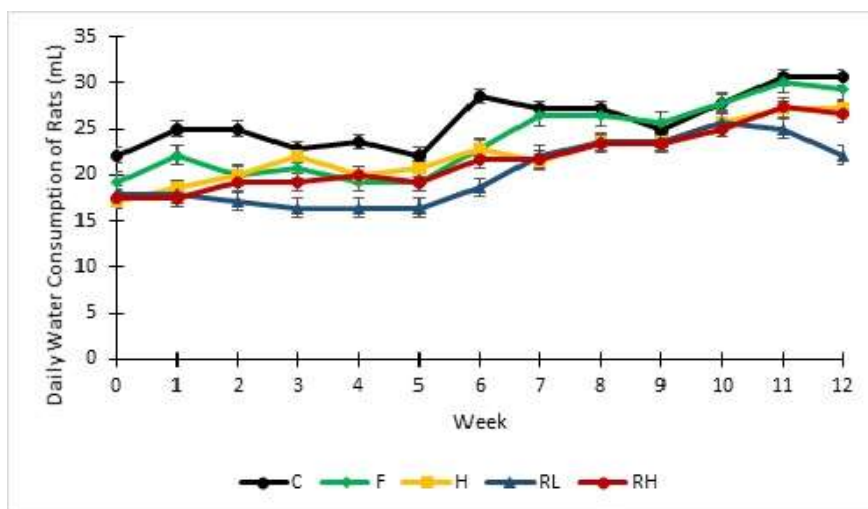


Figure 2: Variation in daily water consumption of rats treated with de-ionized water and test water ad-libitum for 90 days. Data are presented as mean $\pm$ SD (N=7). C- Control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L

In this study, serum creatinine was used as a marker of renal function in rats. The Tukey's pairwise comparisons indicate that the differences in the increment of the serum creatinine level of the rat groups F and H were not significantly different from the control ($p>0.05$) while the RL and RH groups were significantly different ($p<0.05$). The magnitude of the increment in serum creatinine levels in group F, H, RL and RH were 64.65%, 59.60%, 85.49% and 80.56% respectively at the end of the experiment. According to the results, the serum creatinine levels increased notably in RL and RH than the F and H groups in the present study (Figure 3).

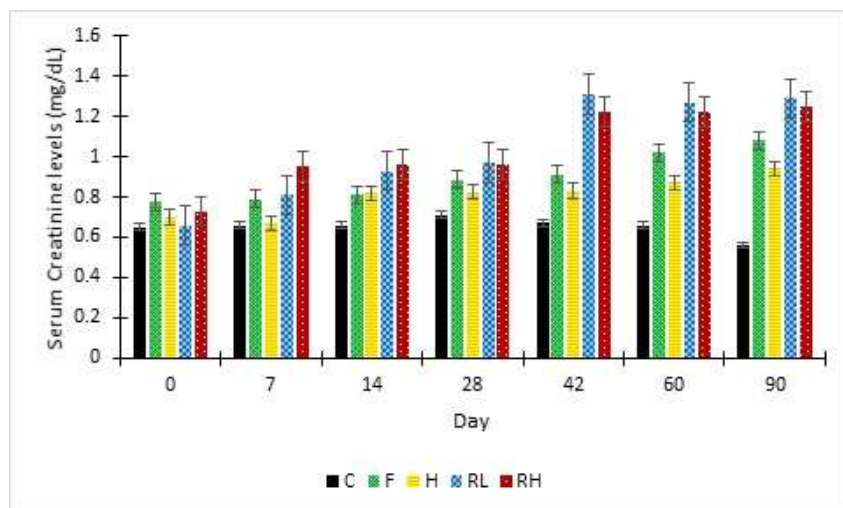


Figure 3: Variation in serum creatinine levels of rats treated with de-ionized water and test water ad-libitum for 90 days. Data are presented as mean $\pm$ SD (N=7). C- control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L

Creatinine is a waste product formed when creatine phosphate is broken down in muscle tissue and excreted in urine after limited tubular reabsorption (Brisco *et al.*, 2016). The accumulation of creatinine in the blood can occur due to renal dysfunction, and elevated serum creatinine level is a marker of renal impairment (Al Salhen & Mahmoud, 2016). The results of this study indicate an increment in serum creatinine levels in all the treated groups compared to the control group, suggesting renal dysfunction in the test groups. However, the RL and RH groups had a greater elevation in serum creatinine than the F and H groups, and the rise in the RL and RH groups occurred earlier than in the F and H groups, showing that the RL and RH groups had more severe renal damage. This finding is similar to that of Perera *et al.*, (2020) who examined the nephrotoxic effects of water hardness combined with high fluoride levels. When test groups were given water samples collected from Mihintale, which had a fluoride level of 1.66 mg/L and hardness of 364 mg/L, serum creatinine levels were considerably higher than the control group treated with water samples collected from the Kandy region with a fluoride level of 0.2 mg/L and hardness of 84 mg/L, which were within the standard limits recommended by the WHO. In addition, Thammitiyagodage *et al.*, (2020) obtained similar results in their study. They observed significantly higher serum creatinine levels in rats who were administered test water samples with high fluoride and calcium concentrations collected from a high CKDu prevalent location in the North Central Province, Sri Lanka.

The level of urine creatinine was evaluated in this experiment to determine the degree of renal injury in rats. The Tukey's pairwise comparisons indicate that the differences in the reduction of the urine creatinine level of the test groups F, H, RL and RH groups were significantly different from the control group ($p < 0.05$). The magnitude of the reduction in urine creatinine levels in group F, H, RL and RH were 74.62%, 71.35%, 80.21% and 78.22% respectively at the 90th day of the experiment. Though, there was significant difference in all test groups compared to the control group the highest magnitude of the reduction in urine creatinine was recorded in the RL group indicating prominent effect at the end of the study (Figure 4).

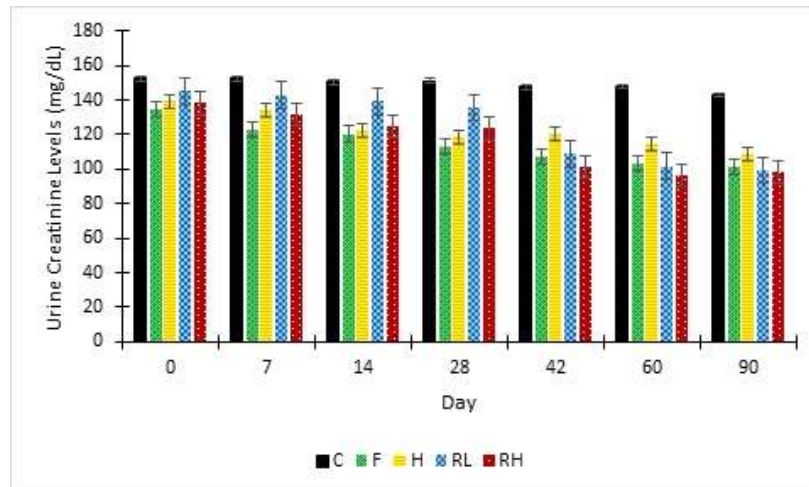


Figure 4: Variation in urine creatinine levels of rats treated with de-ionized water and test water ad-libitum for 90 days. Data are presented as mean $\pm$ SD (N=7). C- control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L

In contrast, decreased urine creatinine levels have been linked to renal impairment (Di Micco *et al.*, 2013). According to the results of the present study, all the treated groups reported decreased urine creatinine levels compared to the control group, with the RL and RH groups demonstrating a more significant reduction than the F and H groups. The relationship between urine creatinine and renal survival in CKD patients were studied by Di Micco *et al.*, (2013), and similar results were found, suggesting a decline in urine creatinine excretion in CKD patients. Furthermore, reduction in urine creatinine excretion rate were observed in the study done by Tynkevich *et al.*, (2014) with 1072 men and 537 women CKD patients.

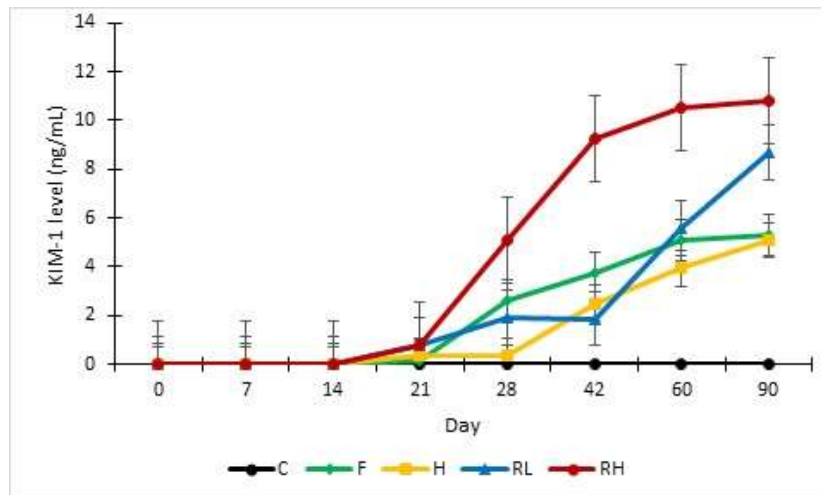


Figure 5: Variation in KIM-1 levels of rats treated with de-ionized water and test water ad-libitum for 90 days. Data are presented as mean $\pm$ SD (N=7). C- control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentration of 1.5:200 mg/L, RH - group for fluoride: hardness concentration of 5:800 mg/L

Kidney injury molecule-1(KIM-1) was measured in this experiment as a marker of renal tubular injury in rats. Elevated KIM-1 levels were recorded in all the test groups from the 21st day of the experiment, while the control

group remained at the baseline. The Tukey's pairwise comparisons indicate that the differences in the increment of the KIM-1 level of the rat groups F, H and RL were not significantly different from the control ($p>0.05$) while the RH group was significantly different ($p<0.05$). According to the results, RH group indicated a more rapid increment in KIM-1 level at the end of the study indicating a renal damage (Figure 5).

Renal KIM-1 levels increase rapidly when there is an acute renal injury, whereas healthy renal tissues do not express KIM-1 (Wu *et al.*, 2018). KIM-1 levels are recognized as a sensitive indicator of renal impairment and it is mostly expressed in inflammatory epithelial cells in the proximal renal tubule (Song *et al.*, 2019). The extracellular fragment of KIM-1 is released into the urine after the renal tubular cell damage and mitogen activated protein kinase signaling pathway controls the shedding of KIM-1 (Zhang *et al.*, 2007). Therefore, KIM-1 levels are recognized as a sensitive indicator of renal impairment and can be used as a biomarker for early detection of renal damage (Cárdenas-González *et al.*, 2013; Song *et al.*, 2019). KIM-1 levels were greater in all of the treated groups than in the control group, with the RH group showing a rapid increase, suggesting early kidney injury. Early renal injury was described by Cárdenas-González *et al.*, (2013) after exposure to environmental fluoride concentrations, as evidenced from the elevated KIM-1 levels. Moreover, research using urine samples from adults and children showed that KIM-1 levels were associated with CKD patients (Carter *et al.*, 2016).

The sections of the renal tissues in the rats belonging to the treated groups showed features of mild renal tubular damage, while sections of the kidneys of the control group had normal histology. The histological alterations were observed in renal tubules in the treated groups F, H, RL, and RH, with the RL and RH groups showing the foremost alterations (Table 2 and Figures 6 to 9).

Table 2: Histopathological evaluations in kidney lesions in rats treated with de-ionized water and test water ad-libitum for 90 days.

Group	Histological features	Control	F	H	RL	RH
Cortex-Tubules	Intraluminal protein	Absent (0)	Mild (1)	Mild (1)	Mild (1)	Mild (1)
	Epithelial alterations- swelling	Absent (0)	Mild (1)	Mild (1)	Mild (1)	Mild (1)
	Nuclear Pyknosis	Absent (0)	Mild (1)	Absent (1)	Mild (1)	Mild (1)
	Vascular Dilatation	Absent (0)	Absent (0)	Absent (0)	Mild (1)	Mild (1)
	Tubular Necrosis	Absent (0)	Absent (0)	Absent (0)	Mild (1)	Absent (0)
	Vascular Congestion	Absent (0)	Absent (0)	Absent (0)	Absent (0)	Mild (1)
Cortex Glomeruli	Mesangium	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
	Glomerular tuft	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
	Bowman's space	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
	Fibrosis	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
Outer medulla	Epithelial alteration- swelling	Absent (0)	Absent (0)	Absent (0)	Absent (0)	Absent (0)
	Intraluminal protein	Absent (0)	Absent (0)	Absent (0)	Absent (0)	Absent (0)
Inner medulla	Intraluminal protein	Absent (0)	Absent (0)	Absent (0)	Absent (0)	Absent (0)
Interstitium	Inflammation	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
	Fibrosis	Normal (0)	Normal (0)	Normal (0)	Normal (0)	Normal (0)

C- control, F - group for 1.5 mg/L fluoride alone, H - group for 200 mg/L hardness alone, RL - group for fluoride: hardness concentrations of 1.5:200 mg/L, RH - group for fluoride: hardness concentrations of 5:800 mg/L (N=7); (0) – normal, (1) – mild, (2) – moderate, (3) – severe

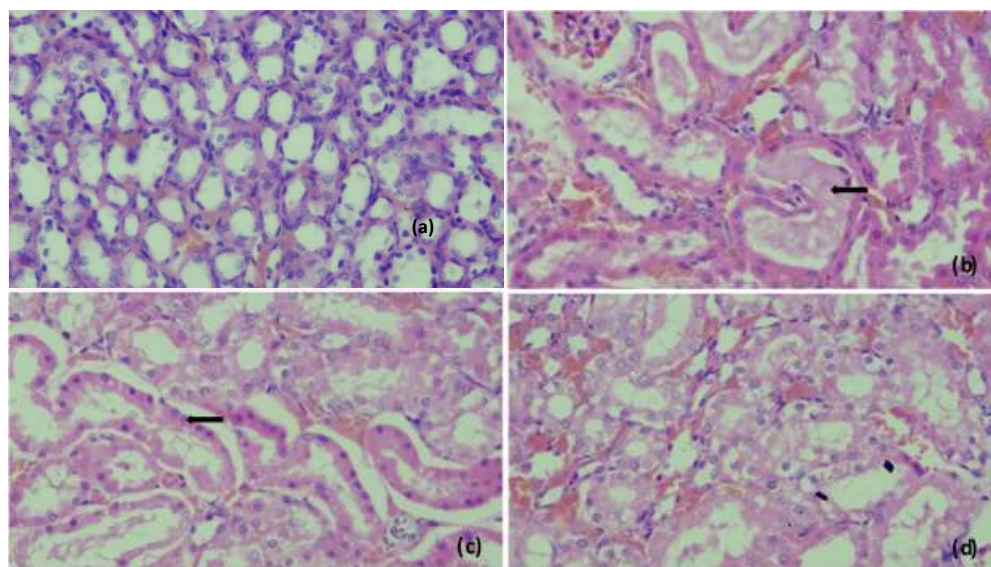


Figure 6: Histopathological evaluations of kidney tissues in 1.5 mg/L fluoride alone group (F) under H & E stain
 a) control (x400), b) Arrow- intraluminal proteins (x400), c) Arrow- cytoplasmic eosinophilia and nuclear pyknosis (x400), d) cellular swelling (x400)

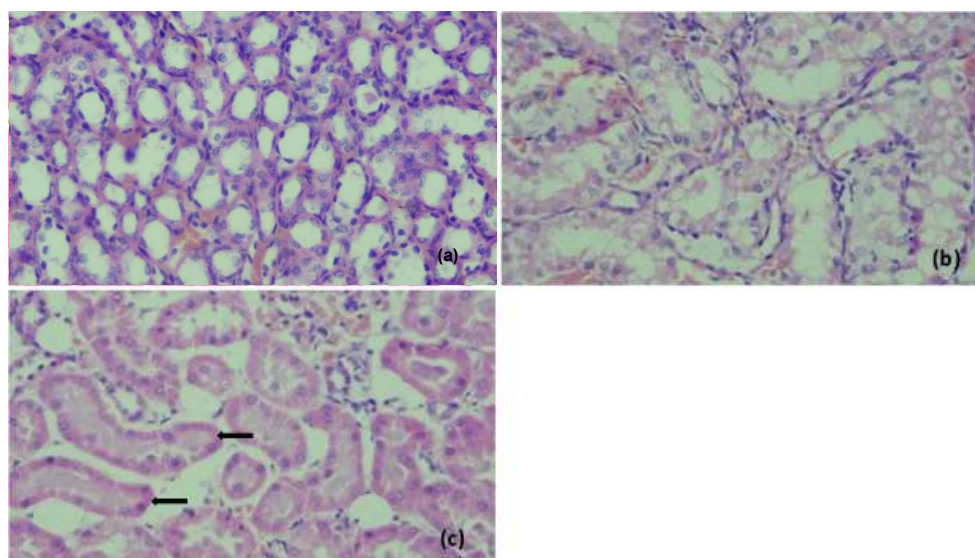


Figure 7: Histopathological evaluations of kidney tissues in 200 mg/L hardness alone group (H) under H & E stain
 a) control (x400) b) cellular swelling (x400), c) Arrow- cytoplasmic eosinophilia (x400)

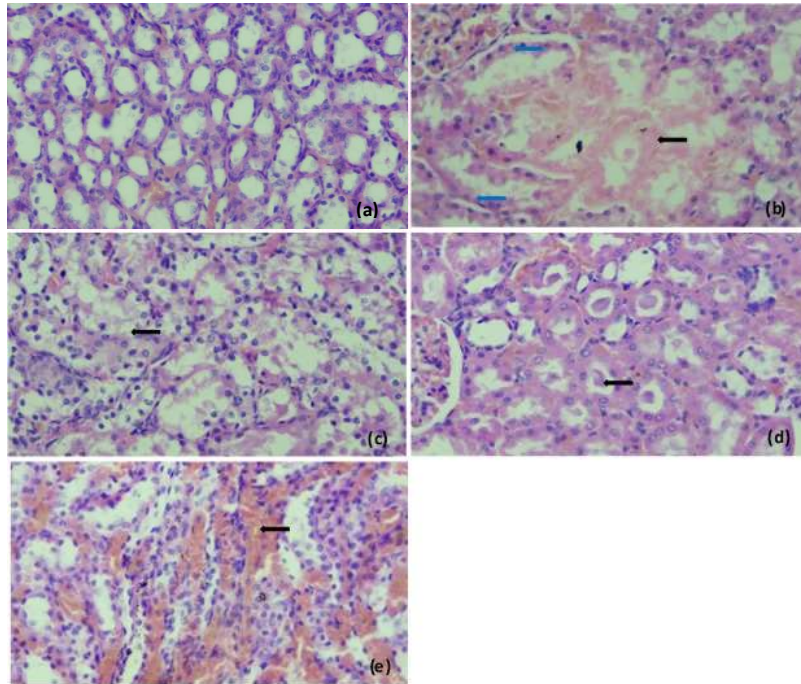


Figure 8: Histopathological evaluations of kidney tissues in 1.5 mg/L fluoride: 200 mg/L hardness concentration group (RL) under H & E stain a) control (x400) b) Arrow (Black) - tubular necrosis (x400), Arrows (Blue) - surrounding tubules with cytoplasmic eosinophilia and nuclear pyknosis c) Arrow- cellular swelling (x400), d) Arrow- intraluminal protein (x400), e) Arrow- vascular dilatation (x400)

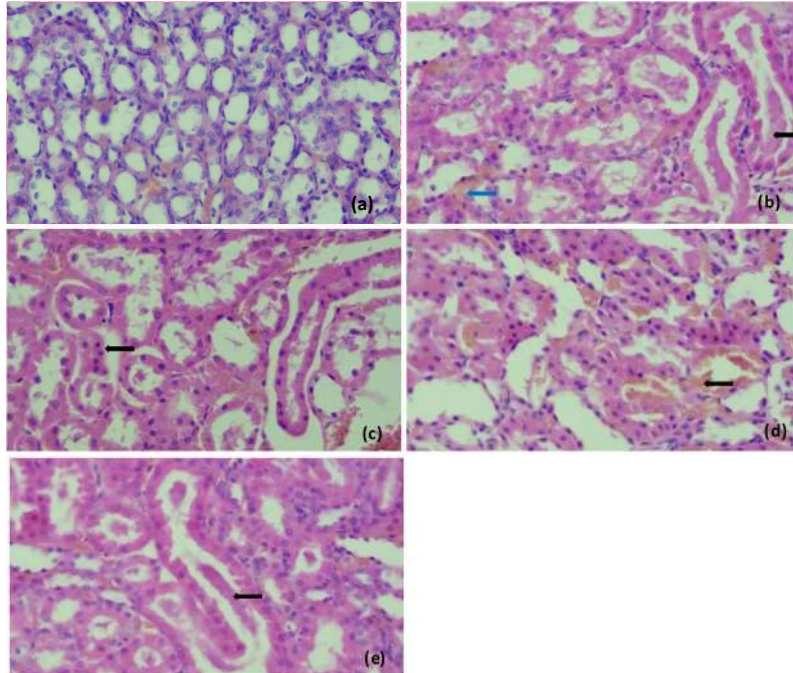


Figure 9: Histopathological evaluations of kidney tissues in 5 mg/L fluoride: 800 mg/L hardness concentration group (RH) under H & E stain a) control (x400), b) Arrow (Black)-intraluminal protein (x400), Arrow (Blue) - vascular dilatation (x400), c) Arrow- nuclear pyknosis (x400), d) Arrow- vascular dilatation (x400), e) Arrow- intraluminal protein (x400)

Histopathological alterations were observed in the renal tubules. Glomeruli lesions such as global glomerular sclerosis, focal glomerular sclerosis, and glomerular enlargement were not observed in the rat renal tissues in the present experiment. Furthermore, interstitial inflammation and the presence of plasma cells, which are representative of interstitial nephritis were also not observed in the interstitium of the kidney tissues in the present study. However, tubular lesions such as the presence of intraluminal proteins, vascular dilatation, cellular swelling, cytoplasmic eosinophilia, nuclear pyknosis, and focal tubular necrosis were observed as histopathological changes in the kidney tubules in the treated groups (F, H, RL, and RH) compared to the control group (Figures 6 to 9).

Similarly, the findings of the histopathology in the study done by Thammitiyagodage *et al.*, (2020) discovered a significantly high index of renal tubular lesions including tubular degeneration and regeneration in the kidneys of the rats that were treated with water collected from the North Central province, which have recorded high hardness and fluoride concentrations compared to the rats treated with water collected from Colombo. The study done by Perera *et al.*, (2020) revealed that there were several histopathological changes in the kidneys, of rats belonging to the groups which were given Mihintale water samples that had fluoride level of 1.66 mg/L and hardness of 364 mg/L. The observed histopathological changes can be listed as loss of brush border, epithelial degeneration in renal tubules. However, the control group treated with distilled water was not exhibited such morphological changes in renal tissues of rats in their study (Perera *et al.*, 2020).

CONCLUSION

The combined effect of fluoride and hardness in drinking water observed in the combination groups (RL and RH) of rats may contribute to more severe impairment of kidneys than the effects of either fluoride or hardness alone (F and H groups of rats). Even though fluoride has nephrotoxic effects, its combined action with hardness may be more prominent. The results of the current study indicate that the high fluoride and hardness concentration in drinking water may be a significant contributory factor to the aetiology of CKDu in Sri Lanka. It is recommended to conduct further testing with more exposure combinations of fluoride and hardness in an animal model to confirm a possible synergistic effect.

Conflict of interest

All the authors state that there is no competing financial interests or personal relationships that may influence the research work reported here.

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