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Impact of Well Water Hardness and Fluoride on Chronic Kidney Disease of Unknown Aetiology in Sri Lanka– Zebrafish as an Animal Model

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ABSTRACT

Chronic kidney disease of unknown etiology (CKDu) is a significant health problem in Sri Lanka. Several hypotheses, such as soil geology, pesticide exposure, cyanotoxins, and prolonged dehydration, have been suggested as possible contributors to CKDu; however, the precise etiology remains unclear. This study aimed to investigate the potential impact of groundwater hardness and fluoride content on CKDu using zebrafish (*Danio rerio*) as an animal model. Sixty well water samples were randomly collected during the dry season from CKDu endemic regions in Sri Lanka, including Galnewa, Rajanganaya, and Medirigiriya in the North Central Province, Dehiattakandiya in the Eastern Province, and Agunukolapelassa (non-endemic area) in the Southern Province as a control. Water pH, conductivity, and dissolved oxygen were measured onsite and within the Sri Lankan Drinking Water Quality Standards (SLS 614: 2013). The highest mean water hardness (285.2 ppm) was recorded in Medirigiriya, and the highest mean fluoride (1.24 ppm) was recorded in Dehiattakandiya, both of which are identified as CKDu high-prevalence areas. The acute toxic effects of these parameters were assessed using zebrafish (*Danio rerio*) embryos. Ninety embryos were exposed up to 96 h post-fertilization (hpf) to water samples representing the highest and lowest water hardness and fluoride concentrations, as single and combined solutions. Embryonic mortality rate in combined exposure (highest hardness and fluoride) was 49.0±0.58%, while hardness alone was 16.7±0.58% and fluoride alone was 18.8±1.15%. Statistical analysis revealed a significant synergistic effect of hardness and fluoride combination ($p < 0.05$) on mortality compared to single exposure. Subsequently, combined exposure caused developmental delays (> 65%) and morphological abnormalities, such as bent body axis and yolk sac edema. Fluorescence images indicated that the damaged premature pronephros in the combined exposure group emitted bright green fluorescence compared to that in the single exposure group. Thus, the findings suggest that the synergistic nephrotoxic effect of high water hardness and fluoride can be a prominent cause of CKDu etiology in Sri Lanka.

INTRODUCTION

Chronic kidney disease (CKD) is a significant health concern worldwide, affecting more than 15% of the population (Cockwell & Fisher 2020). Asia, Central America, Africa, and the Middle Eastern communities have experienced a remarkable increase in CKD cases, especially during the last three decades. CKD of unknown etiology (CKDu) has emerged in farming communities in South Asia (Priyadarshani et al. 2023). CKDu is characterized by a gradual loss of kidney function over time, and the exact cause remains debatable. CKDu is considered multifactorial and is linked to various environmental, occupational, and lifestyle factors. In Sri Lanka, the highest prevalence of CKDu has been reported in the North Central Province, extending to the North Western, Uva, and Eastern provinces, particularly among

farmers (Rajapakse et al. 2016). Several hypotheses have been proposed to explain the origin of CKDu in Sri Lanka. Epidemiological data suggest that CKDu is associated with certain agricultural practices, especially rice farming. These farmers often work under hot and humid conditions, which can lead to heat stress and frequent dehydration. Repetitive heat stress and insufficient hydration may affect kidney function over time (Xu et al. 2021).

Additionally, prolonged exposure to agrochemicals, including pesticides and fertilizers used in agricultural practices, could potentially contribute to the origin and progression of CKDu (Priyadarshani et al. 2023). As most of the population in this region predominantly relies on well water for drinking purposes, poor water quality has been proposed as another contributor to CKDu in the dry zone due to the specific soil geology. Elevated water hardness, fluoride, and the presence of heavy metals act as possible triggering factors. Heavy metals, such as cadmium and arsenic, are known to be nephrotoxic (toxic to the kidneys) and can act as progression factors with long-term exposure, either through contaminated water or soil (Kim et al. 2015). Contaminated drinking water sources, either through natural pollutants or agricultural runoff containing agrochemical residues, heavy metals, and other toxins, have been postulated as other risk factors for CKDu (Pinto et al. 2020). Considering the distribution of patients around the country and the number of patients from specific regions, genetic predispositions may increase individual susceptibility to CKDu when combined with environmental stressors (Abeysiri et al. 2024, Friedman 2019). Some studies have discovered that the role of dietary factors, such as low intake of antioxidants and essential nutrients, increases the vulnerability to CKDu (Wimalawansa & Dissanayake 2019). Throughout prolonged exposure, a combination of these hypotheses, including environmental, occupational, and genetic factors, could contribute to the progression of CKDu (Campese 2022, Liyanage 2022).

Despite these numerous hypotheses, the exact cause of CKDu has not yet been established. This study focused on the synergistic effect of water hardness and fluoride as triggering factors for CKDu in Sri Lanka. Endemic CKDu regions overlap with hydrogeochemical zones that contain elevated fluoride levels and hard water. Available data on groundwater sources demonstrate elevated levels of fluoride and hard or very hard water (Dilrukshi et al. 2023a, Levin et al. 2016), which reflect the unique hydrogeochemistry of the region. Districts with high CKDu prevalence, such as Anuradhapura (18.9%) and Polonnaruwa (13.9%), which are in the North Central Province, have recorded high concentrations of both fluoride (Anuradhapura >2 $mg.L^{-1}$, Polonnaruwa >1 $mg.L^{-1}$) and hardness in Anuradhapura (241-300 $mg.L^{-1}$) and Polonnaruwa (181-240 $mg.L^{-1}$) (Gunawardena et et al. 2021, Indika et et al. 2022). Scientists have pointed out that the cytotoxicity effect of the Na^{2+}/Ca^{2+} ratio in water may influence F^- metabolism. The kidney accumulates more fluoride than all other soft tissues, except for the pineal gland (Inkielewicz & Krechniak 2003). Excess fluoride and hardness exposure may contribute to the development of kidney disease, as CaF2 deposits can promote tubular blockages. This study aimed to evaluate the well water hardness and fluoride content covering random sample points of the North Central Province and Eastern Province, and their possible effect on CKDu. There are high-prevalence (Medirigiriya and Dehiattakandiya) and low-prevalence areas (Galnewa and Rajanganaya) within the endemic region due to the mosaic distribution of cases. CKDu non–endemic area (Agunukolapelassa) in the Southern Province was selected to serve as the control region.

Zebrafish (*Danio rerio)* were used as the animal model to assess the acute toxic effects of water hardness and fluoride on early development and kidney function. *Danio rerio* is considered a well-established vertebrate model and has been successfully used in studies for nearly two decades. Zebrafish have been utilized in different research fields, including biology, toxicology, reproductive studies, genetics, and environmental studies (Hoo et al. 2016). They possess some favorable characteristics to be an ideal model organism, including their small size, rapid development with a short life cycle, optical transparency during early development, tractability in forward genetic screens, genetic similarity to humans (69%), and easy maintenance (Mahanayak 2024). Organogenesis occurs at a very early stage (48 hpf) of embryonic development. Owing to their transparency and genetic similarity to humans, zebrafish provide an effective platform for evaluating the effects of exposure components in human diseases. Therefore, zebrafish can be used as a model organism to understand the toxic effects of different water constituents on body organs, especially kidney development and function. In addition to acute toxicity observations, fluorescent dyes can be used to indicate cellular-level organ damage in zebrafish due to their optical clarity (Lee et al. 2019).

Acridine orange (AO) staining is widely used to detect apoptotic or damaged cells, including renal cells, in zebrafish. Acridine orange selectively binds to nucleic acids and emits different fluorescent signals depending on cell integrity (Hung et al. 2022). In cases of kidney damage, AO helps identify regions of cell death (apoptosis) or injury in developing nephrons. Under a fluorescence microscope, healthy cells emit green fluorescence. In contrast, apoptotic or damaged

cells emit bright green or even orange fluorescence due to changes in nucleic acid structure or increased permeability in damaged cells (Ishaque & Al-Rubeai 2004). This technique was applied in the study to identify the damaged pronephros in exposed zebrafish larvae to assess the potential synergistic effects on kidney development and the possible connection to CKDu.

MATERIALS AND METHODS

Environmental Sample Collection and Analysis

Environmental samples were collected from CKDu-endemic and non-endemic regions in Sri Lanka (Fig. 1). Thirty well water samples were collected using a random sampling method from CKDu high- and low-prevalence sampling sites in CKDu-endemic areas. Medirigiriya and Dehiattakandiya were selected as high-prevalence areas, and Galnewa and Rajanganaya were selected as low-prevalence areas. Agunukolapelassa was considered a non-endemic region (control). Water pH, temperature, conductivity, and dissolved oxygen were measured onsite, and 1 L of water from each sample site was transported to the laboratory in pre-cleaned high-density polypropylene bottles for further analysis. Water hardness was measured in the laboratory using the standard EDTA titrimetric method, and Fluoride, Nitrate, Nitrite, Ammonia, and Total phosphate were measured using spectrophotometric methods (APHA 2017).

Solution Preparation

Desired Hardness solutions (572.6, 40.0 ppm) were prepared using 600 ppm $CaCO_3$ and $MgCl_2$ stock solutions, and fluoride solutions (2.33, 0.37 ppm) were prepared using 100 ppm NaF stock solutions. Embryos were exposed to well water samples from the field and synthetically prepared hardness and fluoride solutions simultaneously in the combined exposure.

Zebrafish Breeding and Exposure

A total of 20 female and male zebrafish were kept separately in glass aquaria (length 80 cm, height 50 cm, width 46 cm) at the Animal House of the University of Sri Jayewardenepura, Sri Lanka. Fish were maintained at 27±1 ^{o}C temperature, and a natural dark/light cycle of 12 h was provided with continuous water exchange. The water pH was maintained within 7-8. The fish were conditioned for two weeks and fed twice daily with artificial fish feed and brine shrimp. Brood stock maintenance was performed according to the OECD (2013) guidelines. The spawning cage was made with a fixed wire mesh in the middle to prevent adults from cannibalizing the eggs. Fish were added to the spawning cage immediately before the onset of darkness, the day before the experiment, at a ratio of 2:1 (male to female) for breeding. Spawning and fertilization occurred within 1 h of light onset the following morning. After spawning, the adult fish and the wire mesh were removed, and the embryos were transferred to a Petri dish (60 mm) containing distilled water. Fertilized eggs were selected using an optical microscope in the presence of embryo cleavage after fertilization.

Exposure series of water hardness (572.6 ppm, 40.0 ppm) and fluoride (2.33 ppm, 0.37 ppm) were prepared according to the environmental sample analysis, and 30 embryos per concentration were exposed in 24-well plates within 2 h of post-fertilization (hpf). To obtain the acute toxic effects of water hardness and fluoride, experiments were conducted with single (hardness and fluoride alone) and combined (hardness + fluoride) exposures. Combined exposure experiments were performed using both environmental samples and synthetic solutions. Negative controls with distilled water and positive controls with 4 ppm 2,4-dichloroaniline were conducted simultaneously. The entire experiment was performed in triplicate using three different batches of embryos.

Observations and AO Staining

Mortality rates, hatching rates, heart rate, developmental delays, and morphological abnormalities were recorded every 24 h until 96 hpf using a light microscope (Optika). At 10 days post-fertilization, the larvae were stained with acridine orange to observe organ damage. Embryos were dipped in 10 mL of 100 $\mu g.mL^{-1}$ of Acridine Orange for 1 h, and after that, several times with E3 buffer media (14.61 g NaCl, 0.63 g KCl, 2.43 g $CaCl_2 \cdot 2H_2O$, and 1.99 g $MgSO_4$ in 1 L Milli-Q water) to remove excess dye (Kaufman et al. 2009). The larvae were then mounted on slides and observed under a fluorescence microscope (Zeiss Axio) to evaluate cell apoptosis.

Statistical Analysis

One-way ANOVA was used to evaluate the significant difference between the fluoride and hardness, single exposures and mortality rates of the zebrafish. Two-way ANOVA was used to evaluate the significant differences in fluoride, hardness, combined exposure, and mortality rates. If $p < 0.05$, the null hypothesis was rejected, which means that there was a statistically significant difference in embryonic mortality and different concentrations of the solutions, and a significant difference between single and combined exposures. Fluorescence intensity was measured to quantify the severity of cell apoptosis using the mean gray value of the fluorescent regions by ImageJ software.

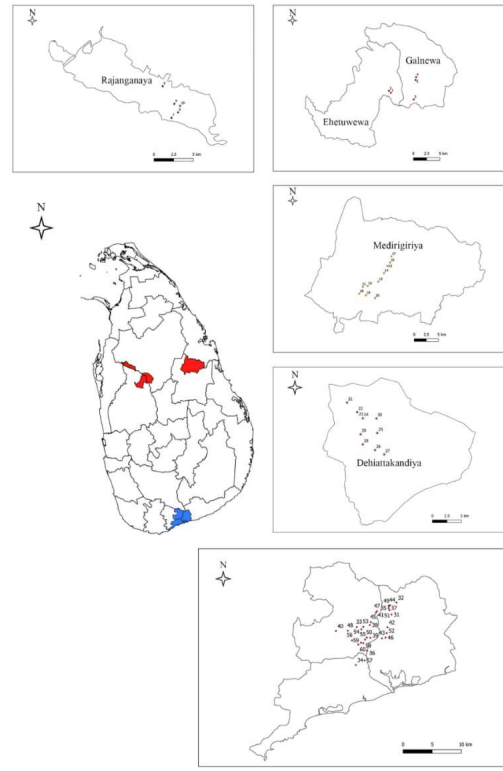


Fig.1: Map of the sampling locations.

Table 1: Hardness and Fluoride concentrations in well water samples in CKDu endemic areas (mean±SD).

Sample No.	Area	Hardness [mg.L^{-1}]	Fluoride [mg.L^{-1}]	Sample No.	Area	Hardness [mg.L^{-1}]	Fluoride [mg.L^{-1}]
1	Galnewa (CKDu low prevalence)	300.00±0.58	0.37±0.00	16	Medirigiriya (CKDu high prevalance)	164.00±0.58	1.33±0.00
2		160.00±0.58	0.53±0.00	17		248.00±0.58	1.32±0.00
3		92.00±0.58	0.86±0.00	18		220.00±0.00	1.21±0.00
4		124.00±0.00	0.72±0.00	19		572.60±0.58	0.45±0.00
5		268.00±0.00	0.54±0.00	20		296.00±0.58	0.52±0.00
6	Rajanganaya (CKDu low prevalence)	260.00±0.00	0.81±0.00	21	Dehiattakan-diya (CKDu high prevalence)	232.00±0.58	1.71±0.00
7		160.00±0.58	0.61±0.00	22		360.00±0.00	0.65±0.00
8		240.00±0.00	0.51±0.00	23		140.00±0.58	0.56±0.00
9		280.00±0.58	2.33±0.00	24		160.00±0.00	0.76±0.00
10		260.00±0.58	0.38±0.00	25		224.00±0.58	1.35±0.00
11	Medirigiriya (CKDu high prevalance)	164.00±0.58	1.04±0.00	26		460.00±0.00	1.17±0.00
12		368.00±0.00	0.88±0.00	27		140.00±0.58	2.06±0.00
13		280.00±0.00	1.97±0.00	28		272.00±0.00	1.57±0.00
14		440.00±0.58	1.80±0.00	29		240.00±0.58	0.70±0.00
15		100.00±0.58	1.26±0.00	30		181.33±0.00	1.87±0.00

RESULTS

Thirty well water samples were randomly collected from drinking water wells from CKDu endemic areas: Galnewa and Rajanganaya had a low prevalence, and Medirigiriya and Dehiattakandiya had a high prevalence. The Agunukolapelassa area was selected as the CKDu non-endemic area, and 30 water samples were collected. Fig. 1 shows the sampling location map.

The highest water hardness was measured as 572.60±0.58 ppm in Medirigiriya, and the lowest was 40.00±0.58 ppm in Dehiattakandiya (Table 1). The highest mean water hardness was recorded as 285.2 ppm in Medirigiriya, and the highest mean fluoride was recorded in Dehiattakandiya as 1.24 ppm, which are identified as CKDu high-prevalence areas.

Agunukolapelassa, where no CKDu patients are recorded, has low water hardness and fluoride compared to CKDu-endemic regions (high and low prevalence) (Table 2). The highest hardness was recorded in the Yakagala area (158.67 ± 0.06 ppm), while the lowest was in Barawakumbuka (16.00 ± 0.06 ppm). Fluoride levels were also lower and remained within the WHO guidelines. Sites with both high hardness and fluoride levels were not identified in this area.

Water pH, conductivity, and dissolved oxygen were measured on-site and were within the Sri Lankan Drinking Water Quality Standards (SLS 614:2013) (Table 3).

The results of the single exposure showed a mortality rate of less than 20% in each case (Table 4). Fluoride had a higher effect on embryonic mortality than hardness, as it caused 18.85% death out of 90 exposed embryos. The hatching rate was affected more by hardness than by fluoride, as it was reduced by more than 10%. A single exposure did not induce any morphological abnormalities or growth retardation.

Table 5 shows the results of the synergistic exposure to water hardness and fluoride on zebrafish embryos. Mortality rates increased with higher concentrations of hardness and fluoride compared to single exposures. Exposed embryos showed more than 50% mortality in both environmental and synthetic solutions at the highest hardness-fluoride combination. However, in the single exposure, it was less than 20%. Hatching rates were severely affected by high water hardness and fluoride, as it resulted in 0% larvae out of embryos after 54 hpf, while in the control, the hatching rate was 83.3%

Fig. 2 illustrates the embryonic developmental stages of zebrafish after exposure. Morphological abnormalities [2(e)], such as edema and bent body axis, were observed in approximately 15% of the larvae at the highest combined exposure. At the same time, they showed less swimming ability and body movements than the other exposures and the control. Low concentrations of combined exposure, single exposure, and control larvae showed no abnormalities after 96 hpf.

Mortality rates at every 24 h interval showed a significant difference between single and synergistic exposures. Mortality rates were higher after 48 hpf and 72 hpf than at 24 hpf and reduced at 96 hpf (Fig. 3). Most internal organs, including the pronephros, become functional at approximately 48 h post-fertilization (hpf). The combined effect of water hardness and fluoride was more fatal at

Table 2**:** Hardness and Fluoride concentrations in well water samples in the non-endemic area of Angunukolapelassa (mean±SD).

Sample No.	Hardness	Fluoride	Sample No.	Hardness	Fluoride
1	98.67±0.06	0.79±0.00	16	120.00±0.00	0.56±0.00
2	158.67±0.06	0.56±0.00	17	61.33±0.06	0.83±0.00
3	60.00±0.06	0.32±0.00	18	53.33±0.06	0.58±0.00
4	53.33±0.06	0.53±0.00	19	42.67±0.00	1.18±0.00
5	114.67±0.00	0.22±0.00	20	62.67±0.06	0.38±0.00
6	32.00±0.06	0.47±0.00	21	16.00±0.06	0.32±0.00
7	29.33±0.00	0.18±0.00	22	25.33±0.06	0.50±0.00
8	22.67±0.06	0.41±0.00	23	41.33±0.06	0.79±0.00
9	24.67±0.06	1.16±0.00	24	54.67±0.06	0.31±0.00
10	140.00±0.06	0.16±0.00	25	114.67±0.06	0.75±0.00
11	85.33±0.06	0.55±0.00	26	68.00±0.06	0.90±0.00
12	58.67±0.06	0.25±0.00	27	81.33±0.06	0.41±0.00
13	93.33±0.06	0.93±0.00	28	45.33±0.06	0.28±0.00
14	62.67±0.06	0.69±0.00	29	81.33±0.06	0.56±0.00
15	78.67±0.06	0.54±0.00	30	60.00±0.06	0.55±0.00

Table 3: Water quality parameters of well water samples from CKDu endemic areas (Galnewa, Rajanganaya, Medirigiriya, and Dehiattakandiya).

Sample No.	Water temperature (°C)	Water pH	Conductivity [µS. cm^{-1}]	DO [$mg.L^{-1}$]	Nitrate [$mg.L^{-1}$]	Nitrite [$mg.L^{-1}$]	Ammonia [$mg.L^{-1}$]	Total phosphate [$mg.L^{-1}$]
1	24.8	7.22	5520	4.87	4.45±0.01	ND	ND	4.41±0.01
2	25.9	7.53	743.9	8.49	4.27±0.02	ND	ND	6.12±0.01
3	28	7.05	721.3	4.18	4.11±0.01	ND	ND	5.73±0.01
4	24.3	7.67	1335	6.49	3.95±0.01	ND	ND	0.63±0.01
5	25.6	7.5	1371	1.17	2.63±0.01	0.016±0.00	ND	6.43±0.02
6	26.3	7.27	465.2	4.87	2.82±0.02	0.248±0.00	ND	6.64±0.01
7	27.5	7.09	829.1	3.55	2.51±0.01	0.014±0.00	ND	8.22±0.01
8	25.8	6.62	908.5	7.58	1.89±0.01	ND	ND	8.32±0.03
9	26.3	5.52	1067	7.2	1.98±0.01	0.013±0.00	ND	8.08±0.01
10	26.9	6.28	1420	7.43	1.53±0.01	0.012±0.00	ND	8.08±0.01
11	24.8	7.45	1703	4.37	1.79±0.01	0.018±0.00	ND	8.60±0.02
12	26.1	7.19	1279	2.68	2.12±0.01	ND	ND	8.60±0.01
13	26.4	6.95	395.4	2.26	0.00±0.00	ND	ND	8.46±0.01
14	26.2	7.18	675	1.62	1.08±0.01	0.031±0.00	ND	6.99±0.02
15	26.9	7.36	483.9	5.77	1.64±0.01	0.014±0.00	ND	3.29±0.01
16	24.9	7.26	939.6	5.99	1.93±0.01	0.019±0.00	ND	8.95±0.01
17	26.4	7.19	541	3.13	2.09±0.01	ND	ND	6.64±0.01
18	27.2	7.06	461	2.52	2.16±0.01	ND	ND	7.48±0.03
19	25.7	7.99	775.4	5.89	2.08±0.02	ND	ND	7.76±0.01
20	26.5	7.49	988	2.71	2.04±0.01	ND	ND	2.59±0.01
21	26.0	7.47	827.1	3.6	0.18±0.01	ND	ND	8.39±0.01
22	26.8	6.76	442.3	3.7	0.67±0.02	ND	ND	0.14±0.04
23	25.8	7.13	310.3	3.87	1.87±0.01	ND	ND	0.14±0.01
24	26.3	6.79	280.4	2.48	1.88±0.01	0.023±0.00	ND	0.29±0.01
25	25.6	7.29	311.3	2.1	0.78±0.01	0.024±0.00	ND	0.14±0.02
26	26.0	7.39	361.9	4.06	0.00±0.01	0.014±0.00	ND	2.59±0.01
27	26.6	7.11	376.9	3.04	2.04±0.01	ND	ND	6.99±0.02
28	25.7	7.21	245	4.21	1.53±0.01	ND	ND	8.22±0.01
29	26.4	6.88	289.7	4.75	3.95±0.01	ND	ND	5.73±0.01
30	27.1	7.05	780.3	2.45	2.09±0.01	ND	ND	2.59±0.01

Table 4: Embryonic acute toxicity observations of single exposure to water hardness and fluoride.

Sample	Mortality rate%	Hatching rate% [54 hpf]	Heart rate [bpm] [72hpf]
572.6ppm (H)	16.7±0.58	26.7	136±4
40.0ppm (H)	10.0±1.15	46.7	140±6
2.33 ppm (F)	18.8±1.15	40.0	150±8
0.37ppm (F)	13.3±1.15	66.7	152±4
Control	0	83.3	140±2

Table 5: Embryonic observations to combined exposure of water hardness and fluoride (ES- Environmental sample, SS- Synthetic solution, H- Water Hardness, F- Fluoride).

Solution [ppm]	Mortality rate%		Hatching rate% [54hpf]		Heart rate [bpm] [72 hpf]	
	ES	SS	ES	SS	ES	SS
572.6 ppm (H) + 2.33 ppm (F)	48.2±1.15	49.0±0.58	0	0	164±4	160±2
572.6 ppm (H) + 0.37 ppm (F)	32.4±0.58	36.7±1.15	0	0	165±6	158±2
40.0 ppm (H) + 2.33 ppm (F)	40.3±0.00	43.3±0.58	6.6	10.0	141±8	144±4
40.0 ppm (H) + 0.37 ppm (F)	19.8±1.15	23.3±1.15	16.6	23.3	127±4	115±2
Control	0		83.3		144±2	

2 (a) 2(b) 2(c) 2(d) 2 (e)

Fig. 2: Light microscope images of embryonic development after exposure 2(a) 2 hpf, 2(b) 24 hpf, 2(c) 48 hpf, 2(d) 72 hpf, 2(e) embryo with bent body axis and yolk sack edema (10×4).

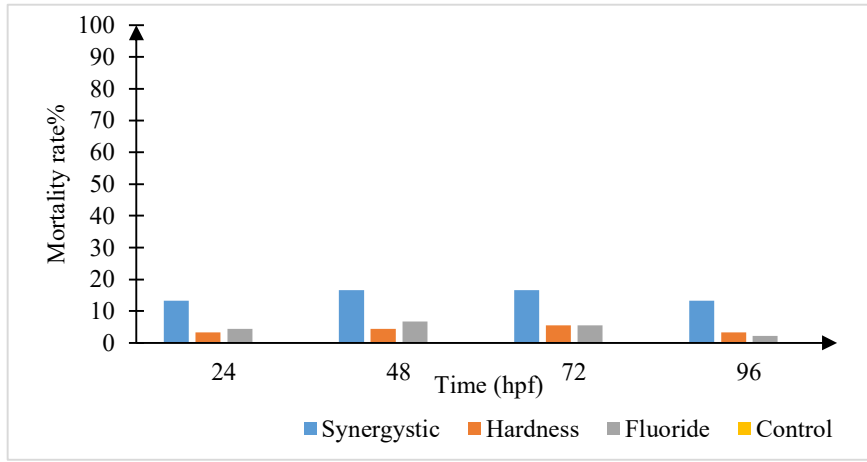
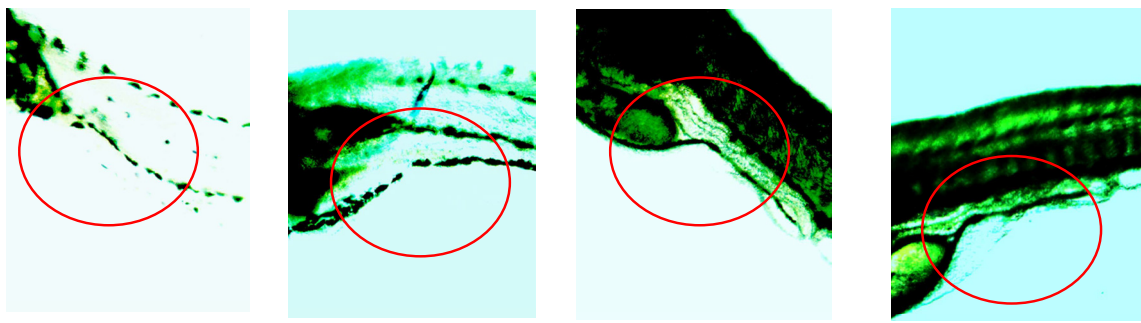


Fig. 3: Comparison of mortality rates of embryos exposed to single and synergistic exposures over time (hpf).

each stage, whereas fluoride exhibited a more lethal effect than water hardness alone. The control group exhibited no mortality throughout the experiment.

One-way ANOVA showed a significant effect ($p < 0.05$) of both single and combined exposure on embryonic mortality. Two-way ANOVA comparison indicated a significant difference ($p < 0.05$) between single and combined exposure, as combined treatments (hardness + fluoride) caused significantly higher mortality than individual treatments. Subsequently, Pearson's correlation showed a weak positive correlation ($r = 0.3$) for single exposures of hardness, fluoride, and mortality rates, while a moderate positive correlation ($r = 0.5$) for combined exposure and mortality rates.

The pronephros is a paired organ that filters blood, which is composed of lymphoid tissue, adrenocortical cells, and the pronephric duct. Fluorescence microscopic images



4(a) 4 (b) 4(c) 4(d)

Fig. 4: Fluorescence microscopic images of the AO staining larvae (a) Control, (b) Hardness single exposure, (c) Fluoride single exposure, (d) Combined hardness and fluoride exposure.

(Fig. 4) indicated kidney cell apoptosis after Acridine Orange staining. It filters blood circulation and is responsible for osmoregulation during larval life. After staining, the larvae in the control group showed no bright green emissions in the pronephros area. At the same time, single hardness [4(b)], single fluoride [4(c)], and combined exposure [4(d)] showed increasing bright green emissions in the pronephros located in the embryo's trunk region. Fluorescence intensity was measured as the mean gray value of the fluorescent regions, which were 146, 190, and 204, respectively. According to the degree and pattern of fluorescence, these emissions correlate with the severity of kidney injury.

DISCUSSION

CKDu is defined as chronic kidney damage or dysfunction without conventional risk factors such as diabetes, hypertension, or genetic conditions. The exact cause of CKDu remains unclear, but several factors are suspected to contribute to its etiology. According to recent research findings, high levels of calcium and magnesium in water (hard water) are potential risk factors (Wasana et al. 2016). Elevated fluoride levels in drinking water have been studied as potential contributors to CKDu, with concerns about fluoride accumulating in the kidneys over time (Liyanage et al. 2022). North Central Province in Sri Lanka is a hotspot for CKDu, where water hardness and fluoride levels exceed the WHO water quality guidelines in most drinking-water wells. According to Yang et al. (2022), local groundwater in a CKDu prevalent area of Sri Lanka could cause kidney damage, implying that high water hardness and fluorine might be the inducible environmental factors for the etiological cause of CKDu. Therefore, the synergistic effect of water hardness and fluoride may cause the progression of CKDu among communities using drinking-water wells. In this study, the fluoride level reached 2.33 ppm, which is the highest, and the mean value was 0.69 ppm, which is higher than that in other regions in Sri Lanka. According to the WHO drinking water guidelines, the maximum level of fluoride is 1.5 ppm, and the study area has recorded more than seven sites with fluoride levels exceeding this limit. According to the water quality parameters, the water hardness ranged at the very hard water level. These parameters have alarming values that can lead to CKDu in endemic areas, with their synergistic effect on kidney function. CKDu non-endemic area water samples did not exceed the WHO maximum value (<1.5 ppm) but recorded very hard water values. This also suggests that a synergistic effect could act as a triggering factor for CKDu in the country.

In this study, the synergistic effects of water hardness and fluoride were evaluated using zebrafish embryos and larvae. Animal models can be used to assess the acute toxic effects of drinking water and determine kidney damage at the pronephric level. Zebrafish share approximately 70% of their genes with humans, and their nephron structures (the basic functional unit of the kidney) are remarkably similar, making them a good model for studying renal diseases. Zebrafish embryos can be used to test various environmental substances to determine their nephrotoxic potential, aiding in the identification of potential CKDu triggers. The study data suggest a significant relationship ($p< 0.05$) between elevated water hardness levels and fluoride with kidney damage in the exposed embryos. Both environmental and synthetic solutions showed mortality in over 50% of the embryos and morphological abnormalities, such as yolk sac edema and curved body axis, which are expressed due to kidney damage. Single exposures to fluoride and hardness revealed less acute toxicity in embryos than combined exposures. Fluoride alone had a more lethal effect on embryonic development than hardness alone. The role of the kidneys can be explained, the body's primary excretory organs in regulating the metabolism of poisons and foreign toxins. According to studies, the kidneys filter and reabsorb 50–80% of the fluoride that the body consumes (Dharmaratne 2019), which causes abnormal development and cell death in the embryonic stages. Fluoride-induced kidney structural damage can inhibit renal cell proliferation when exposed to higher concentrations for extended periods (Wimalawansa 2020).

Hard water mainly consists of Ca^{2+} and Mg^{2+} ions. Ca^{2+} plays various roles in the composition of bones and teeth and can control nerve transmission and material release (Salama & Mohammed 2023). Many previous studies have found that F^- and Ca^{2+} antagonistically affect biology. Once F^- is absorbed by the body, it enters the blood to form insoluble CaF_2 precipitates and deposits in the kidney tissue while being excreted (Nóbrega et al. 2019). When a combination of hardness and fluoride is exposed to zebrafish embryos for a certain period, it accumulates CaF_2 in the kidney as precipitates, which can ultimately cause tubular damage and renal failure in adult zebrafish. This can be identified externally by the edema conditions of the larvae after hatching. Edema is a pathological condition characterized by abnormal fluid accumulation in the body (Wimalawansa, 2020). Zebrafish depend on their skin, kidneys, and gills to maintain proper internal water volume and osmotic pressure. Kidneys are essential for controlling ion and water levels for appropriate osmoregulation. As the kidney is responsible for waste elimination in fish, any dysfunction can lead to fluid retention (Wang et al. 2024). This expanded the larvae's body shape and exhibited edema under a light microscope.

The mortality of embryos is higher after 48 hpf, when larvae start to form vital organs in the body. The pronephros, the sole kidney of zebrafish larvae, develops during embryogenesis and is fully formed approximately 48 h after fertilization (Serluca & Fishman 2001). Accelerating the lethal effect at this stage can be due to the damage of vital organs by the exposed synergistic solutions. The pronephros is highly sensitive and accumulates more CaF_2 at this stage, which can cause tubular degeneration. Long-term exposure to well water with elevated hardness and fluoride can have a similar effect on human kidneys. The origin and progression of CKDu are triggered by well water from the CKDu prevalence area, which recorded higher water hardness and fluoride levels exceeding the WHO guidelines.

Pronephros damage was confirmed using acridine orange staining. Acridine orange (AO) staining is a common technique used to detect apoptotic or damaged cells, including kidney cells, in zebrafish embryos. Acridine orange selectively binds to nucleic acids and emits different fluorescent signals depending on cell integrity. In cases of kidney damage, AO helps identify regions of cell death (apoptosis) or injury in developing nephrons (Plemel et al. 2017). Under a microscope, damaged cells emit bright green fluorescence due to changes in nucleic acid structure or increased permeability in damaged cells (2017). The degree and pattern of fluorescence correlated with the severity of kidney injury and apoptosis. The highest emission was observed after combined hardness and fluoride solution exposure, as it recorded the highest mean gray value (204). A single exposure to fluoride (190) had a higher green emission intensity than a single exposure to hardness (146), suggesting that fluoride has a more significant effect on kidney damage than hard water. Hardness triggers the impact of fluoride on kidney tubules when combined, and induces prolonged renal damage (Dilrukshi et al. 2023), causing higher emissions of fluorescence due to cell apoptosis.

Most farming communities in the North Central Province depend on deep or shallow wells for drinking and other purposes. Due to the soil geology of the area, the water contains elevated levels of Ca^{2+}, $Mg^{2+,}$ and F^- ions. Continued exposure to very hard water and high fluoride levels may be the primary reason for the unknown etiology of Chronic Kidney Disease in Sri Lanka. Dissanayake (2005) suggested that the fluoride concentration and its interactions with other ions, such as Ca^{2+}, $Mg^{2+,}$ and $Na^{2+,}$ could trigger the onset of CKDu in Sri Lanka. According to Wickramarathna et al. (2017), hardness reached values as high as 516 $mg.L^{-1}$ in some groundwater in CKDu-affected regions. The mean hardness of groundwater in all samples collected during the pre-monsoon period was 181 $mg.L^{-1}$ Fig. 3, indicating very hard water.

In this study, the highest hardness was recorded as 572 ppm, and the mean hardness was 251 ppm, indicating very hard water according to the WHO guidelines. Fluoride was also found at alarming levels in most wells in the area, exceeding the safe drinking water levels. Individuals consume water with high hardness and fluoride, which can accumulate CaF_2 in kidney tissues with long-term exposure. Similar to zebrafish kidney damage, human kidneys are affected by continuous deposition, leading to oxidative stress, inflammation, and tubular damage. Patients with CKDu often show signs of proximal tubular injury, which aligns with the effects of fluoride toxicity. In particular, farming communities are exposed to high temperatures and reduced kidney perfusion, which exacerbates fluoride retention. This study proposes that the combination of water hardness and fluoride is one of the triggering factors behind Chronic Kidney Disease of unknown aetiology in Sri Lanka. To mitigate CKDu progression among communities, several actions can be taken, as this has become a severe health concern in the country. Routine water testing in high-risk areas, continuous monitoring, and regulation of groundwater wells in the North Central, Eastern, and Central Provinces must be implemented to overcome this issue. Most areas have reverse osmosis water systems and filter units at the household level. People should be encouraged to use treated water rather than well water by providing adequate awareness of the risk of CKDu. Agricultural practices and the use of agrochemicals must be monitored, and safety measures are needed. Most importantly, early detection of the disease and affordable treatments are essential as long-term prevention strategies.

CONCLUSIONS

The study data revealed a significant relationship between water hardness and fluoride levels in CKDu in Sri Lanka. Both parameters exceeded the WHO drinking water guidelines in most wells consumed by the affected community. Zebrafish were used as an animal model to express embryonic and cellular-level damage to the organs. Morphological abnormalities and mortality rates were positively correlated with exposure to water hardness and fluoride concentrations. Combined exposure to hardness and fluoride resulted in more apoptotic cells than other exposures, with a higher degree of emissions in the kidney area. Hence, prolonged exposure to the combination of hardness and fluoride and its synergistic effect can be one of the prominent causes of CKDu progression, as it causes renal cell damage.

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