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Cytogenotoxicity of drinking water of selected dug wells in an area where chronic kidney disease of unknown aetiology is prevalent in Sri Lanka

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Abstract Consumption of contaminated groundwater is implicated as a risk factor for chronic kidney disease of unknown aetiology (CKDu), which is a public health problem in some tropical countries, including Sri Lanka. However, the application of bioanalytical tools to assess the potential toxicity of groundwater consumed by CKDu-affected communities has received limited attention. This study explored for the first time the potential cytogenotoxic risk of drinking water from selected dug wells in a CKDu endemic area in Sri Lanka using *in vivo* bioassays (*Cyprinus carpio* erythrocyte-based and *Allium cepa* root meristem-based tests), complementary to physicochemical analysis. In dug well water used by households with CKDu, fluoride levels in dry and rainy periods, and electrical conductivity, alkalinity, and total hardness levels in the dry period were significantly higher than the well water used by households with no CKDu cases in the same endemic area. Exposure to water of dug wells used by households with CKDu triggered nuclear buds and fragmented nuclei induction in fish erythrocytes and mitosis suppression and induction of condensed nuclei and binuclei in root meristems in most cases. Kidneys of fish exposed to dug well water exhibited renal tubular degeneration. Bioassays revealed cytogenotoxicity of dug well water, which may be due to interactive toxicity of contaminant mixtures including fluoride and other unknown toxicants. Untreated water from such dug wells may pose cytogenotoxic risks to the consumers, influencing disease progression.

Keywords: CKDu, Dug well water quality, Fluoride contamination, Bioanalytical tool, Drinking water interactive toxicity, Cytogenotoxicity

INTRODUCTION

Chronic kidney disease of unknown aetiology (CKDu) has become an increasingly concerning public health problem in recent decades, particularly in some tropical countries in Central America and South Asia, including Sri Lanka (Weaver et al., 2015; Pearce et al., 2019; Priyadarshani et al., 2023). CKDu cannot be attributed to diabetes, hypertension or chronic glomerulonephritis, which are commonly recognised risk factors for chronic kidney disease. However, CKDu is a slowly progressing nephropathy which is irreversible (Weaver et al., 2015; Priyadarshani et al., 2023). Although various hypotheses have been proposed regarding the causative factor of CKDu, its aetiology appears to be multifactorial, with no single biological or hydrogeochemical parameter directly related to the disease (Levin et al., 2016; Wimalawansa, 2016). CKDu is highly prevalent in agricultural villages in some administrative districts in the dry zone of Sri Lanka, especially in the North Central Province, causing a severe public health problem among poor rural communities (Kafle et al., 2019; Ranasinghe et al., 2019). Epidemiological studies revealed that the majority of the CKDu patients had consumed untreated groundwater mostly from shallow dug wells (Jayasekara et al. 2015). Although the cause of CKDu in affected populations is uncertain, chronic dietary exposure to untreated groundwater in CKDu-impacted regions is hypothesised as a likely risk factor of the disease (Hettithanthri et al., 2021; Imbulana & Oguma, 2021).

Groundwater quality assessments in CKDu-related studies have mostly focused on the comparison of hydrogeochemical parameters such as heavy metals, hardness and fluoride in CKDu endemic and non-endemic areas in Sri Lanka with the available drinking water quality standards (Wickramarathna et al., 2017; Sandanayake et al., 2023; Chandrajith et al., 2024). In addition to hydrogeochemical analysis, the safety of drinking water sources can be assessed using biological effect-based methods, such as bioassays (Dingemans et al., 2018; Pronk et al., 2024), which can evaluate interactive toxicity among mixtures of bioavailable contaminants in groundwater originating from natural and anthropogenic sources. Interaction of contaminant mixtures in the groundwater in CKDu prevalent areas may induce cytotoxic and genotoxic effects on consumers, causing cellular damage and cell death, which can lead to organ damage posing health risks. As biological models, mammals are the best choice for assessing the *in vivo* toxic potential of drinking water for predicting human health risks. However, practical and ethical constraints limit the use of higher vertebrates as model organisms. Therefore, as an initial step, plants and lower vertebrates can be used as biological models to identify potential toxic hazards of groundwater used in the CKDu endemic areas for predicting health risks. Fish serve as a popular vertebrate model in scientific research due to their genetic closeness to humans and ease of study. The erythrocytic abnormality test in fish is a useful *in vivo* bioassay for screening cytotoxic/genotoxic potential of water sources (Hemachandra & Pathiratne, 2017a, b; Canedo et al., 2021). Moreover, the *Allium cepa* root meristem-based test is a simple, cost-effective and sensitive *in vivo* plant bioassay which shows similarity with mammalian test systems (Leme & Marin-Morales, 2009; Bonciu et al., 2018). The cytogenotoxic potential of drinking water sources has recently been assessed using root meristem-based tests with *A. cepa* (Hemachandra & Pathiratne, 2017a; Weerasekara et al., 2024).

In CKDu-endemic areas in Sri Lanka, wells used by families with clinically confirmed CKDu patients are irregularly interspersed among wells used by families without CKDu (Balasooriya et al., 2020). It is not known whether the water quality of dug wells used by households with CKDu patients for drinking poses greater health risks than that of wells used by households without CKDu in the same endemic area. The objective of this study was to explore the potential cytogenotoxic risk of untreated water of domestic dug wells used by a group of households with CKDu cases in a CKDu endemic area and domestic dug wells used by a group of households with no CKDu cases in the same endemic area by cytogenetic bioassays complementary to conventional physicochemical analysis. Short-term *in vivo* cytogenetic bioassays using two model organisms, the fish *Cyprinus carpio* (Common carp) and the plant, *A. cepa* (Common onion), were used for screening potential cytogenotoxicity of untreated water of the selected dug wells in the endemic area. Additionally, the histological structure of the kidneys of the fish exposed to dug well water was examined to assess any exposure-induced alterations.

MATERIALS AND METHODS

Study area and selected dug wells

In Sri Lanka, the prevalence of CKDu is high in the divisional secretariat of Medawachchiya (Gunatilake et al., 2014), which is located in the dry zone of the country, where prolonged dry periods prevail due to low rainfall. The area receives limited precipitation during the northeast monsoon, typically from October to February. Drinking water sources in rural communities in the Medawachchiya area are mostly dug wells. In addition, for drinking purposes, untreated water from tube wells and springs is also used by some families. Our field survey covering 340 drinking water dug wells in Medawachchiya showed that most wells are shallow (≤9 m) and lined with cement or brick walls. We observed that the dug wells used by families with clinically confirmed CKDu cases for drinking and the dug wells used by other families with no CKDu cases are erratically located in the Medawachchiya area. Of the 340 dug wells surveyed, untreated water from 107 wells had been used as a drinking water source by households with confirmed CKDu cases in the area. However, most households with CKDu cases currently use filtered water for drinking, as community-based reverse osmosis plants have been established to provide safe drinking water. Yet, our survey revealed that untreated water from 20 dug wells in the area was used as the only drinking water source by several families, which included households with CKDu cases as well as households with no cases of CKDu. Of these 20 dug wells, eight domestic dug wells, which had been used as the only water source for both drinking and cooking purposes by the families for >10 years, were selected in this study by judgmental sampling (Fig. 1) for exploring the potential cytogenotoxicity of dug well water by bioassays. The eight selected dug wells included four used by households with clinically confirmed CKDu cases based on medical reports (with at least two adults per family) and four dug wells used by households without a CKDu diagnosis. Both groups of households were residents of the Medawachchiya area for at least 10 years. Locations and the size of the selected dug wells are presented in Table 1. The remaining 12 dug wells had to be excluded from this study for two main reasons: some wells had been used as the sole source of drinking water for less than 10 years, and/or some wells were shared by multiple families in the area (family with one CKDu case and families with no confirmed CKDu cases) for drinking or cooking purposes.

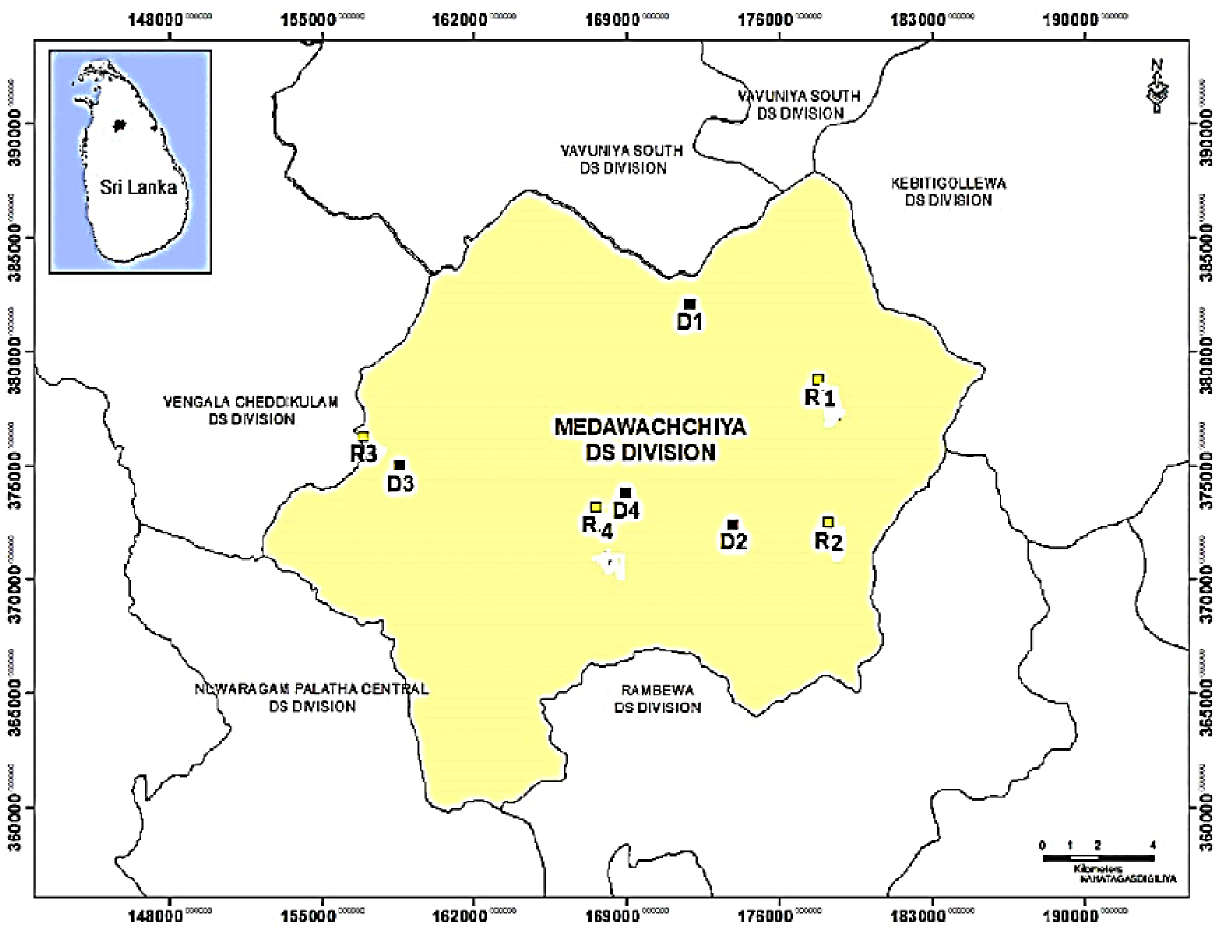

Fig 1. Schematic illustration of selected dug wells in Medawachchiya Divisional Secretariat (DS), Sri Lanka used for drinking water quality analysis. D1, D2, D3 and D4 represent the dug wells used by households with CKDu cases. R1, R2, R3 and R4 represent the dug wells used by households with no CKDu cases.

Table 1. Size and location of the selected drinking water dug wells used for water sampling

Type of dug well and code	Size of the dug well		Village in Medawachchiya
	Depth (m)	Diameter (m)	
Wells of CKDu households			
D1	9.1	2.3	Dutuwewa
D2	9.1	2.4	Lanadiulwewa
D3	7.6	2.4	Yakawewa
D4	9.1	3.7	Ihalathammennawa
Wells of households with no CKDu cases			
R1	5.8	1.8	Ethakada
R2	6.4	2.4	Gallelegama
R3	9.6	2.6	Mahasiyabalagaskada
R4	13.7	2.7	Isinbassagama

Sampling of dug well water

Water samples were collected from the selected domestic dug wells during the dry period in the pre-monsoon season (July 2016) and during the slight precipitation period (October 2017) in the monsoon season (Fig. 2). In each sampling event, samples of water were collected in duplicates from the selected dug wells into clean polypropylene bottles which had been soaked overnight with 10% nitric acid and rinsed with distilled water several times to remove the impurities. Before sample collection, the bottles were rinsed with dug well water. Separate sets of bottles were used to collect the samples for analysis of different physicochemical parameters in the water of each dug well. Water samples collected for analysis of heavy metals (loid)s were acidified to pH=2 on site with the concentrated nitric acid. Water samples were transported to the laboratory under ice-cold storage conditions and refrigerated at 4°C until chemical parameter measurements were conducted. Simultaneously, samples of water from each dug well (40 L) were transported to the laboratory in transparent polythene bags under ice-cold storage conditions for bioassay. The water was used for bioassay on the following day after allowing it to reach room temperature (27-29°C).

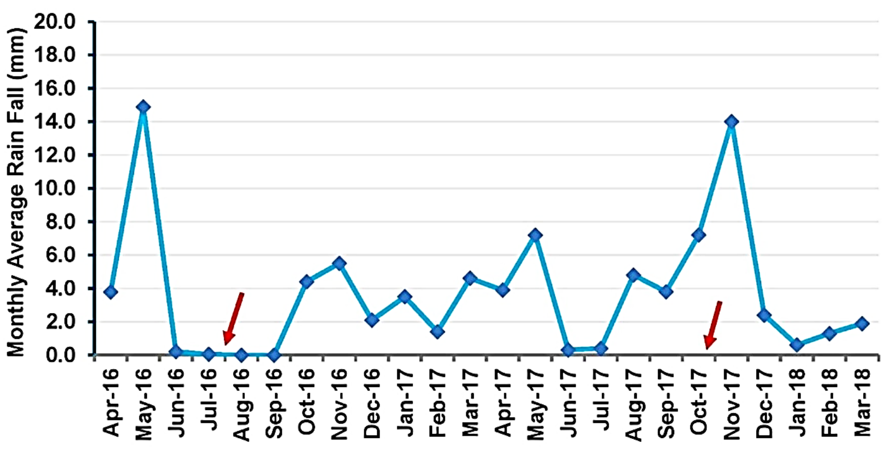

Fig 2. Monthly average rainfall of Anuradhapura, Sri Lanka (close to Medawachchiya area) during the period April 2016 to March 2018.

Physicochemical parameters in well water and control exposure media

In situ parameters (pH, temperature, and electrical conductivity) of the well water were measured on site using potable water quality meters. As described in APHA (2012), alkalinity (method 2320B), total hardness (method 2340C), fluoride (method 4500FC), chloride (method 4500 Cl B), ammoniacal-N (method 4500 NH3 F. Phenate), nitrate (method 4500 NO3- E) and phosphate (method 4500PE) levels in the well water samples were analysed in the laboratory within two days of the sample collection. Selected heavy metal(loid)s namely arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) in the acidified well water samples were analysed in the Industrial Technology Institute, Sri Lanka. Metal(loid) analysis was conducted using Inductively Coupled Plasma - Mass Spectrometry (Agilent 7900 ICP-MS) following Method 200.8 of USEPA (1994). Limits of detection for analysis of As, Cd, Pb and Hg (mean + 10 SD of the blank) were 1, 1, 10 and 1 µg/L, respectively. All physicochemical analyses were performed adhering to quality assurance and quality control procedures, including calibration of the instruments following the manufacturer's instructions before use, use of analytical grade reagents for analyses, use of reference standards for calibration and replicate analysis. Concurrently, physicochemical parameters of the control exposure media used for bioassays: Control 1: potable drinking water (source: natural spring) and Control 2: aged tap water (source: treated river water for drinking) were measured following the same methods stated for analysis of well water samples.

Cytogenetic bioassays

The plant bioassay was performed with *A. cepa* bulbs placed in glass test tubes filled with water samples from the eight dug wells and two control waters (Control 1: potable water from a natural spring, Control 2: aged tap water) as the test exposure media (n=10 onion bulbs per medium). Five bulbs were randomly taken at the end of 48 hours of exposure from each exposure medium, and root tips from each onion bulb were fixed overnight at 4°C in ethanol: glacial acetic acid (3:1 v/v) medium. For microscopic analysis, the root tips were processed as described earlier (Pathiratne et al., 2015). A slide stained with acetocarmine was prepared from each bulb (n=5 onion bulbs from each exposure medium). The slides were coded blindly, and the stained meristematic cells were observed under the light microscope for scoring dividing cells and the occurrence of micronuclei and specific nuclear abnormalities in the cells. From each slide, at least one thousand cells were examined. The mitotic index for each onion bulb was calculated as the percentage of dividing cells relative to the total number of cells counted per slide. The frequency of specific nuclear abnormalities was expressed as the number of cells with specific abnormality from a total of 1000 cells counted from root meristems of each onion bulb (Pathiratne et al., 2015).

For the fish bioassay, *C. carpio* fingerlings were bought from a national culture collection and acclimated to laboratory conditions for two weeks in aerated aged tap water. We followed applicable international ethical guidelines (EU Directive 2010/63/EU for animal experiments and the U.K. Animals [Scientific Procedures] Act, 1986 and associated guidelines) for the care and use of animals in the experiments when using fish for bioassay. Acclimated fish in groups (25 L, n=10 fish per exposure medium) were introduced to separate glass tanks filled with samples of dug well water, potable water from a natural spring (Control 1) and aged tap water (Control 2) for 5 days. The tanks were aerated continuously. The pH, temperature, and dissolved oxygen concentrations of the exposed water ranged from 6.8–8.0, 27–28°C, and 5.6–6.1 mg/L, respectively. Mortality of fish was not observed in any of the tanks during the five-day exposure. Fish (n = 5 per exposure medium) were euthanised with benzocaine before blood samples were collected from the caudal vein at the end of the five-day exposure period. Blood smears prepared on glass slides were stained with Giemsa. Abnormalities in fish erythrocytes were identified and quantified under a light microscope in more than 1,000 erythrocytes per fish (Barsiene et al., 2006). Data were presented as the number of cells exhibiting each abnormality per 1,000 erythrocytes from each fish.

Histopathological assessment of fish kidneys

Samples of fish fingerlings (8-12cm in length) exposed to dug well water and control water for five days were dissected, and pieces of posterior kidneys were preserved in 10% neutral buffered formalin, dehydrated using ethanol and chloroform and embedded in paraffin wax (Bucke, 1989). Histological sections (5 µm) were cut using a rotary microtome. Slides stained with hematoxylin and eosin, were examined under a light microscope to assess any alterations in kidney tissues (Hibia, 1982). Histological alterations in the kidney were quantified by calculating the 'histopathological index' based on different types of histopathological alterations in the fish kidney as described by Bernet et al. (1999). For a specific alteration seen in the kidney tissue of each fish, quantification was based on extension of a pathological change (rated with a 'score value' ranging from 0 to 6, depending on the degree and the extent of the alteration) and its pathological importance (defined as an 'importance factor' ranging from 1 to 3 depending on the reversibility or irreversibility of the change). Ten areas of the kidney tissue on the slide were randomly selected for the analysis. For each fish, the kidney histopathological index (I) was calculated using the equation, $I = \sum(a \times W)$ where, a=score value for specific alteration pattern and W=importance factor specific alteration (Bernet et al., 1999).

Statistical analysis

Minitab Software (version 17) was used in the statistical analysis. For each physicochemical parameter in dug well water during a given sampling event, potential significant differences between wells used by CKDu households and those used by non-CKDu households were assessed using the nonparametric Mann-Whitney U test. The same test was used separately for each category of wells to find any significant differences in the physico-chemical parameters between the dry period and the slightly rainy period. For bioassays, all the data relevant to cytogenotoxicity assessment and histopathological assessment were transformed into log10 (x+1), as some data sets had null values. Transformed toxicity data relevant to water samples from each dug well were compared with each other using a one-way Analysis of Variance (ANOVA) test. Multiple comparisons of the means were carried out using Tukey's test. The Anderson–Darling test was used to assess the normality of the data sets prior to performing ANOVA. In all cases, statistical significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

In most CKDu-related studies, the quality of groundwater in CKDu-endemic areas of Sri Lanka has been compared with that in non-endemic areas and/or with potable water, using hydrogeochemical parameters to explore potential links between CKDu prevalence and drinking water quality (i.e. Wickramarathna et al., 2017; Sandanayake et al., 2023; Chandrajith et al., 2024). However, less attention has been paid to utilising bioanalytical tools for evaluating the potential toxicity of groundwater used by the CKDu-affected communities for drinking. In a water quality assessment of deep tube wells in a CKDu endemic area, Weerasekara et al. (2024) emphasised the importance of using bioassays as complementary tools for drinking water quality assessments in CKDu hotspots. As the majority of the CKDu patients had consumed untreated groundwater mostly from shallow dug wells in the country (Jayasekara et al., 2015), this study explored for the first time the potential interactive toxicity of contaminant mixtures in shallow dug well waters in a CKDu endemic area using biological effect-based methods. Complementary to physicochemical characteristics, we compared, for the first time, the quality of drinking water from dug wells used exclusively by CKDu-affected households with that from wells used exclusively by non-CKDu households in the same endemic area, based on potential cytogenotoxic effects using two *in vivo* bioassays (*C. carpio* erythrocyte-based and *A. cepa* root meristem-based tests). Further, the potential nephrotoxic effects of dug well water were assessed by examining histological alterations in the kidneys of *C. carpio* exposed to untreated dug well water.

Physicochemical characteristics of dug well water

Quality of groundwater in the dug wells can be deteriorated by elevated mineralisation due to geochemical processes. In addition, the quality of groundwater can be changed by recharging and discharging rates of aquifers, together with evaporation and rainfall (Banyikwa, 2023). Of the analysed physicochemical parameters (Table 2) in water from the selected dug wells in Medawachchiya, most parameters, including pH, chloride and nitrate levels, comply with the national standards for potable drinking water in both sampling events (SLSI 2013). Arsenic, cadmium, lead, and mercury in the water of the dug wells were below the limit of detection with one exception (Table 2). Arsenic was detected (2 μg/L) in the drinking water of one dug well used by a CKDu-affected household (D1 well) during the dry period. Nevertheless, it was much below the standard/guideline set for arsenic (10 μg/L) in potable drinking water (SLSI 2013; WHO 2017). The involvement of heavy metal(loid)s such as arsenic, cadmium, and lead in drinking water as aetiological factors of CKDu has not been supported by some hydrogeochemical investigations on CKDu endemic areas in Sri Lanka (Wickramarathna, et al. 2017; Herath, et al. 2018). Our heavy metal(loid) data from drinking water in a limited number of dug wells in the Medawachchiya area also corroborate these findings. Yet, in most cases, alkalinity and total hardness levels (Table 2) in the dug well waters exceeded the respective limits for Sri Lanka standards (200 mg/L and 250 mg/L, respectively) for potable drinking water (SLSI 2013). Elevated levels of total hardness in the dug well water could be due to metamorphic aquifers with rich calcium and magnesium-containing minerals in this area (Chandrajith et al., 2011). Interestingly, the total hardness level was markedly lower in one dug well used by a non-CKDu household (R4 well) compared with the other dug wells in both sampling events (Table 2). Our results indicate the possibility of the occurrence of dug wells with moderately hard water in nearby areas of high hardwater dug wells in the same CKDu endemic area. With respect to fluoride, a wide variation of fluoride levels in different dug wells was seen in our study in the slightly rainy period (0.1-3.4 mg/L) compared to the dry period (1.01-2.56 mg/L). Exceedance of national standards of fluoride in drinking water (1 mg/L) was seen in the water of all dug wells in the dry period and the water of one dug well (D2 well) in the slightly rainy period (Table 2). High fluoride levels in dug well waters could be due to leaching of fluoride ions into groundwater due to intense weathering of fluoride-containing rocks and minerals in the hot climatic season in the dry zone of Sri Lanka (Chandrajith et al., 2020).

Compared with CKDu non-endemic areas in Sri Lanka, certain groundwater quality parameters particularly total hardness, alkalinity, and fluoride were generally higher in CKDu-endemic areas (Hettithanthri et al., 2021; Imbulana & Oguma, 2021). In this study, comparison of detected physicochemical parameters in drinking water from the two groups of dug wells (Table 3) revealed that electrical conductivity, alkalinity, total hardness and fluoride levels in the drinking water of the dug wells used by households with CKDu cases were significantly greater than the levels of respective parameters in the water from dug wells used by households with no CKDu cases in the same CKDu endemic area in the dry period ($P < 0.05$). Although mean values of most parameters in the drinking water from dug wells used by CKDu-affected households were higher during the slightly rainy period, only the fluoride level was significantly greater ($P < 0.05$) than that of the dug well water used by households with no CKDu cases (Table 3). High electrical conductivity of the stagnant dug well water indicates elevated ionicity due to longer resident time of contaminants in hard rock aquifers. High ionicity may be associated with elevated alkalinity (i.e. bicarbonate and carbonate ions), total hardness (calcium, magnesium and bicarbonate ions) and fluoride ion levels in addition to the anions such as phosphate, nitrate and chloride, and other dissolved cations (i.e. potassium and sodium ions) in the dug well water. Significantly higher levels of fluoride in the dug well water used by households with CKDu cases in comparison to dug well water used by households with no CKDu cases in both sampling events (Table 3) may imply a potential link between high fluoride levels in their drinking water and the occurrence of CKDu. A recent study also demonstrated significantly higher fluoride concentrations in groundwater samples related to CKDu than those in groundwater samples related to non-CKDu (mean values: 0.80 mg/L and 0.45 mg/L, respectively), indicating that fluoride is a potential risk factor causing CKDu (Shi et al., 2023). Comparisons of physicochemical data in the dry and slightly rainy periods (Table 3) indicate no statistically significant seasonal differences with respect to mean fluoride levels in the water of the households with CKDu cases, whereas mean fluoride level in the water of the non-CKDu wells were significantly much lower ($P < 0.05$) in the slightly rainy period compared to the dry period. Yet, electrical conductivity and chloride levels in the water of both groups of wells were significantly higher ($P < 0.05$) in the dry period than those levels in the slightly rainy period. High chloride levels could have also contributed to elevated levels of ionicity in dug well waters during the dry period. Compared with the dry period, ammoniacal-N levels were significantly elevated ($P < 0.05$) in the water from both groups of wells during the slightly rainy period (Table 3). Moreover, the mean phosphate level in water from non-CKDu wells was significantly higher in the slightly rainy period ($P < 0.05$) compared to the dry period. In the dug well water, sources of phosphate and ammoniacal-N may be from agricultural activities in the area. Differences observed in some physicochemical parameters in the water of shallow CKDu-related dug wells and non-CKDu dug wells may be associated with evaporation and precipitation, as well as discharged and recharged rates of different aquifers located even in the same CKDu endemic area. Dry climate conditions prevailed in the first sampling period might have induced specific changes in the water level in the aquifers, influencing the drinking water quality of the shallow dug wells in the area.

Table 2. Comparison of physicochemical parameters* in the untreated drinking water from the selected domestic dug wells, potable drinking water (spring water) and tap water with drinking water quality standards

Water source	pH	EC (µS/cm)	Alkalinity (mg/L)	Total Hardness (mg/L)	Fluoride (mg/L)	Chloride (mg/L)	Ammoniacal-N (mg/L)	Nitrate (mg/L)	Phosphate (mg/L)	Arsenic (µg/L)
Dry period (July 2016)										
Dug wells										
D1	7.1	903	**378**	**365**	**1.60**	73	0.001	0.001	0.321	2
D2	7.7	864	**383**	**305**	**2.56**	64	0.001	0.042	0.085	<1
D3	7.5	980	**413**	245	**1.63**	60	0.003	0.233	0.055	<1
D4	7.3	761	**293**	**276**	**2.00**	67	0.013	0.136	0.039	<1
R1	6.9	742	178	200	**1.01**	127	0.006	0.001	0.002	<1
R2	8.0	728	**293**	**254**	**1.30**	58	0.001	0.008	0.012	<1
R3	7.3	553	**250**	175	**1.58**	31	0.008	0.582	0.056	<1
R4	6.9	416	185	99	**1.08**	27	0.001	0.014	0.021	<1
Spring water	6.9	75	45	13	<0.1	2	0.05	0.01	0.02	<1
Tap water	7.0	95	38	24	<0.1	5	0.1	0.14	0.01	<1
Period with slight rain (October 2017)										
Dug wells										
D1	7.4	359	**405**	**421**	0.69	8	0.212	0.019	0.267	<1
D2	7.9	352	**320**	**252**	**3.40**	17	0.407	0.036	0.208	<1
D3	7.7	365	**388**	**292**	0.61	5	0.075	0.058	0.173	<1
D4	7.6	350	**315**	**378**	0.97	12	0.102	0.023	0.153	<1
R1	7.7	326	**325**	**337**	0.35	11	0.078	0.084	0.119	<1
R2	7.6	351	**355**	**337**	0.10	7	0.087	0.045	0.122	<1
R3	7.1	330	**253**	**270**	0.66	9	0.065	0.145	0.169	<1
R4	7.0	132	90	62	0.39	1	0.067	0.002	0.106	<1
Spring water	7.2	64	42	23	<0.1	1	0.05	0.01	0.01	<1
Tap water	7.1	61	30	25	<0.1	1	0.16	0.09	0.01	<1
Drinking water quality standards										
SLSI(2013)	6.5-8.5	-	200	250	1.0	250	-	50	-	10
WHO(2017)	-	-	-	-	1.5	-	-	50	-	10

*Cadmium, lead, and mercury levels were below the detection limits (<1, <10 and <1 µg/L respectively). Bold numbers indicate exceedance of the drinking water standards specified by SLSI (2013). D1, D2, D3 and D4 represent the dug wells used by households with clinically confirmed CKDu cases. R1, R2, R3 and R4 represent the dug wells used by other households in the same endemic area. Spring water (potable drinking water) and tap water are control water used for the bioassays (Control1 and Control 2 respectively).

Table 3. Comparison of physicochemical parameters of untreated drinking water from selected dug wells of CKDu households and wells of households with no CKDu cases in the same endemic area

Parameter	Dry period (July 2016)		Period with slight rain (October 2017)	
	Wells of CKDu households	Wells of no CKDu households	Wells of CKDu households	Wells of no CKDu households
Temperature(°C)	28.9±0.5	28.6 ±0.4	27.3±0.4	27.8± 0.5
pH	7.43±0.12	7.26 ±0.26	7.65±0.13	7.37± 0.17
EC(µS/cm)	877 ± 46*[a]	610±78[1]	357±3[b]	285±51[2]
Alkalinity(mg/L)	366±26*	226±27	357± 23	256± 59
Total hardness(mg/L)	298±26*	182± 32	334± 39	252±65
Fluoride(mg/L)	1.95±0.22*	1.24±0.13[1]	1.42±0.67*	0.38 ± 0.12[2]
Chloride(mg/L)	66.0 ±2.7[a]	60.8± 23.1[1]	10.4±2.6[b]	6.5±2.3[2]
Ammoniacal-N(mg/L)	0.005 ± 0.003[a]	0.004 ± 0.002[1]	0.199±0.075[b]	0.074± 0.005[2]
Nitrate(mg/L)	0.103±0.052	0.151±0.144	0.034±0.009	0.069±0.031
Phosphate(mg/L)	0.125 ±0.066	0.023±0.012[1]	0.200± 0.025	0.129± 0.014[2]

Data are presented as mean ± SEM (n=4) for each category of the well per each sampling event. Asterisks indicate that the data relevant to the wells of CKDu households are significantly greater than the wells used by households with no CKDu cases in the specific sampling period. For wells used by CKDu households, the data in a row indicated with different superscript letters are significantly different from each other; For wells used by other households, the data in a row indicated with different superscript numbers are significantly different from each other (Mann-Whitney U test, $P < 0.05$)

Recent hydrogeochemical investigations related to CKDu hotspots in Sri Lanka signify that the association of higher fluoride levels with higher hardness is the main feature of groundwater from CKDu prevalent areas compared to non-CKDu areas (Wickramarathna et al., 2017; Balasooriya et al., 2020; Chandrajith et al., 2024). Physicochemical analysis of selected dug well water in this study revealed that in well water used by households with CKDu cases, electrical conductivity, alkalinity, and total hardness levels in the dry period and fluoride levels in both periods were significantly higher than the well water used by households with no CKDu cases in the same endemic area. Although small amounts of fluoride have beneficial effects, high levels of fluoride entering the human body will affect the proper functioning of various organs, including the kidneys (Wu et al., 2022). It has been hypothesised that the synergistic action of fluoride and other ions in the hard water of shallow, stagnant household wells in CKDu hotspots in Sri Lanka may contribute to kidney damage and disease progression (Wasana et al., 2016; Balasooriya et al., 2020; Imbulana & Oguma, 2021). Physicochemical analysis of this study also supports the hypothesis that the interactive effects of high levels of fluoride and other contaminants found in hard water of shallow dug wells may be associated with CKDu.

Cytogenotoxic potential of dug well water

Physicochemical parameters of the control waters used for cytogenotoxicity bioassays (*C. carpio* erythrocyte-based and *A. cepa* root meristem-based tests) in the two sampling events (Table 2) complied with Sri Lankan drinking water quality standards for potable water (SLSI 2013). Exposure to genotoxic contaminants can induce damage to DNA as well as proteins involved in the cell division process, causing instability of the genome in the cells, which may result in the formation of micronuclei and nuclear abnormalities (Fenech et al., 2020). Fish erythrocytic micronuclei and nuclear abnormalities test is a cost-effective and reliable bioanalytical tool for screening cytogenotoxic potential of water sources (Hemachandra & Pathiratne, 2017a; D'Agostini & Maestra, 2021). In teleost fish, the anterior kidney (or head kidney) is the main erythropoietic organ and depending on the fish species, erythrocytes originating in the kidneys seem to reach the blood circulation after 2 to 4 days (Barsiene et al., 2006). Micronucleus is an abnormal, extra-small nucleus present in erythrocytes in addition to the main nucleus (Canedo et al., 2021). In the present study, micronuclei and several nuclear abnormalities (nuclear buds, notched nuclei and fragmented nuclei) were detected in the erythrocytes of the fish *C. carpio* exposed to untreated dug well water in the CKDu endemic area (Fig. 3). Micronuclei in the erythrocytes were observed in fish exposed to the water from all dug wells used by CKDu households and one dug well used by non-CKDu household (R3 well) in both sampling events (Fig. 4a). However, statistically significant induction of erythrocytic micronuclei compared to the controls was found only in the fish exposed to water of one dug well used by CKDu household (D4 well) in the dry period ($P = 0.05$). In dividing cells, micronuclei can arise from alterations in chromosomal distribution or chromosome structure, as well as from chromosomal breakage during cell division process following exposure to genotoxic contaminants (Fenech et al., 2020; Canedo et al., 2021). Morphological variations in the nucleus of fish erythrocytes, such as nuclear buds or notched nuclei, have also been explained as nuclear lesions related to genetic damage (Canedo et al., 2021). Frequency of nuclear buds in the erythrocytes of the fish exposed to water of most dug wells used by CKDu households was significantly higher ($P \leq 0.05$) than that of the fish exposed to water of dug wells used by non-CKDu households and control waters (Fig. 4b). Nuclear buds can be formed when eliminating amplified nuclear DNA in the cell division and collecting its remnants towards the nuclear periphery (Canedo et al., 2021). Even though the occurrence of erythrocytes with notched nuclei was considerably higher in the erythrocytes exposed to water from the dug wells used by CKDu households and non-CKDu households, the differences were not statistically significant from the controls ($P > 0.05$) due to high variability (results not shown). Yet, frequency of erythrocytes with nuclear fragments was significantly greater ($P \leq 0.05$) in the fish exposed to dug well water used by both groups of households in the dry period compared to the fish exposed to control waters (Fig. 4c). Moreover, in the dry period, a significantly greater induction of erythrocytes with nuclear fragments was found in the fish exposed to well water used by CKDu households compared to the water used by non-CKDu households ($P \leq 0.05$). In the slightly rainy period, significantly higher induction of erythrocytes with nuclear fragments was found only in the fish exposed to dug well water used by CKDu households ($P \leq 0.05$) compared to other fish groups. The occurrence of erythrocytes with nuclear fragmentation (karyorrhexis) suggests an apoptosis process leading to cell death. Greater frequency of erythrocytes with nuclear fragmentation observed in the fish exposed to the water from dug wells used by CKDu households indicates marked cytogenotoxic effects. Greater induction of nuclear buds and nuclear fragmentation in erythrocytes of circulating blood of the fish exposed to dug well water, especially the well water used by CKDu cases, revealed cytogenotoxic effects of the contaminants in their drinking water.

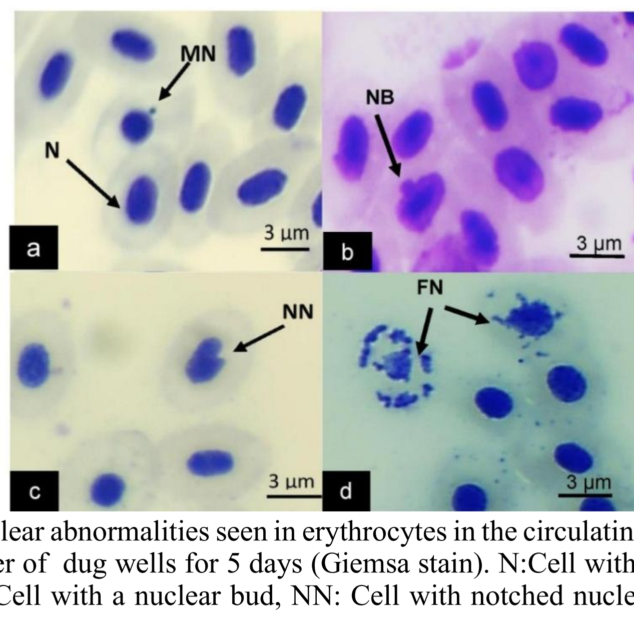

Fig 3. Representative nuclear abnormalities seen in erythrocytes in the circulating blood of *Cyprinus carpio* exposed to untreated water of dug wells for 5 days (Giemsa stain). N:Cell with normal nucleus, MN: Cell with micronucleus, NB: Cell with a nuclear bud, NN: Cell with notched nucleus, FN: Cells with nuclear fragmentations.

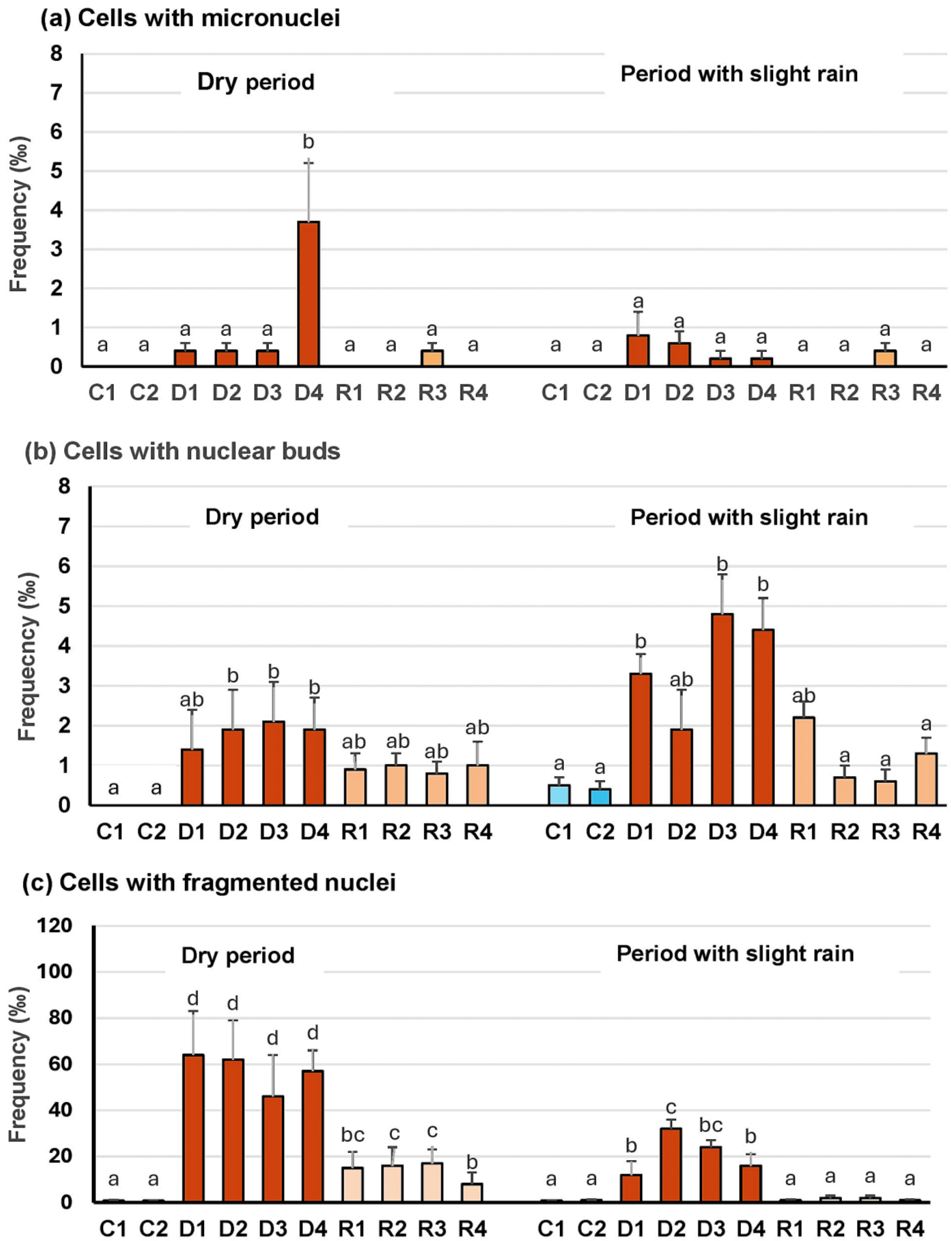

Fig 4. Occurrence of (a) micronuclei, (b) nuclear buds and (c) fragmented nuclei in erythrocytes in the blood of *Cyprinus carpio* following 5 days exposure to potable drinking water from a natural spring (C1), aged tap water (C2) and untreated drinking water samples from selected dug wells used by households with CKDu cases (D1, D2, D3 and D4) and dug wells used by households with no CKDu cases in the same endemic area (R1, R2, R3 and R4). Data are presented as mean ± SEM (n=5). For a specific sampling period, the bars indicated by different letters are significantly different from each other (ANOVA, Tukey's test, $P \leq 0.05$).

A. cepa root meristem-based cytogenetic assay is a low-cost, simple and sensitive *in vivo* bioanalytical tool for screening potential cytotoxicity and genotoxicity of contaminants (Leme & Marin-Morales, 2009; Bonciu et al., 2018). In this test, suppression of mitotic index and induction of condensed nuclei in the root meristem indicate cytotoxicity, whereas induction of micronuclei and nuclear abnormalities such as nuclear buds and binuclei indicate genotoxicity (Leme & Marin-Morales, 2009). In the present study, cells with micronuclei, nuclear buds, condensed nuclei and binuclei were detected in the root tip cells exposed to untreated dug well water (Fig. 5), but the occurrence of cells with micronuclei was very rare. Regarding the frequency of micronuclei and nuclear buds, no statistically significant differences ($P > 0.05$) were observed in the root tip cells exposed to untreated dug well water compared with the respective controls (results not shown). Yet, mitotic indices of the root meristems of the onion bulbs exposed to the water from the three dug wells used by households with CKDu cases (Fig. 6a) were significantly lower than those exposed to the control waters in the dry period ($P \leq 0.05$), indicating obstruction of the progression of the mitotic cell division process. Incidence of the cells with condensed nuclei was significantly higher in the root meristems exposed to well water used by households with CKDu cases compared to the controls ($P \leq 0.05$) in the dry period (Fig. 6b). With respect to mitotic index and condensed nuclei occurrence, statistically significant differences were not observed ($P > 0.05$) among the root meristems exposed to water from wells of households with no CKDu cases and the controls in both sampling events. The occurrence of condensed nuclei as a result of increased condensation of nuclear chromatin in the root tip cells implies damage to the genome (Palmieri et al., 2016). Condensed nuclei in the root tip cells indicate that they are subjected to the apoptosis process, which can cause cell death. In the root meristems exposed to well water used by CKDu-affected households, observed mitosis suppression could be linked to the cell death process as shown by condensed nuclei induction in the root cells. In the root cells, binuclei may occur as a result of the obstruction of the normal cytoplasmic division process after the mitosis (Leme & Marin-Morales, 2009). Frequency of binucleated cells in the root meristems exposed to the water from all the dug wells used by households with CKDu cases were significantly higher ($P \leq 0.05$) than in the controls (Fig. 6c) in the first sampling period, indicating cytogenotoxic effects of the water samples in the dry period. Significant mitotic suppression, along with a high frequency of cells with condensed nuclei and binucleated cells in the root meristems of onion bulbs exposed to water from dug wells used by CKDu-affected households, indicated pronounced cytogenotoxic effects of the contaminants, particularly during the dry period.

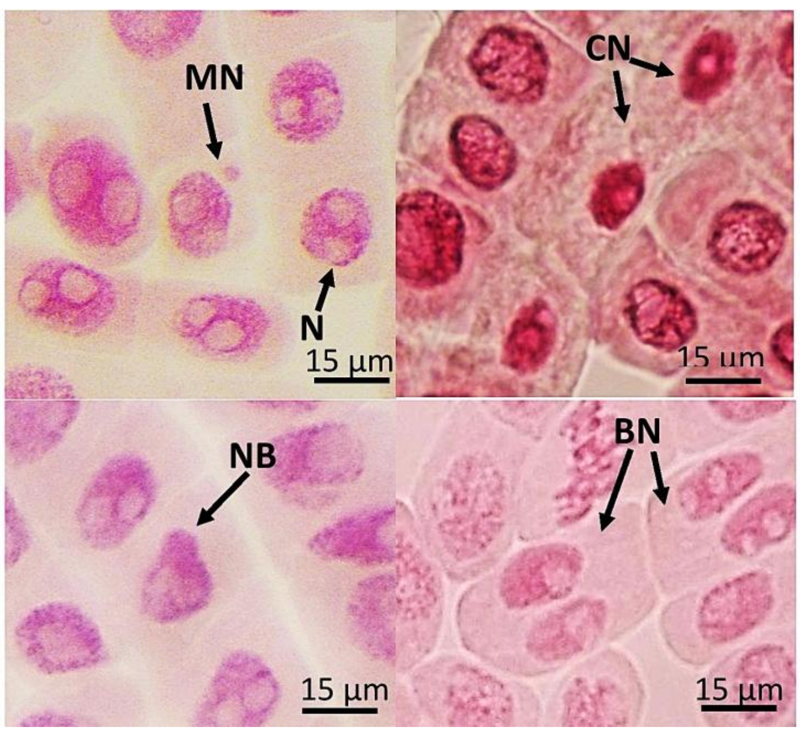

Fig 5. Root meristematic cells in *Allium cepa* exposed to untreated water of dug wells for 48 hours and stained with acetocarmine, showing cells with normal nucleus (N), micronucleus (MN), condensed nuclei (CN), nuclear bud (NB) and binucleated cells (BN).

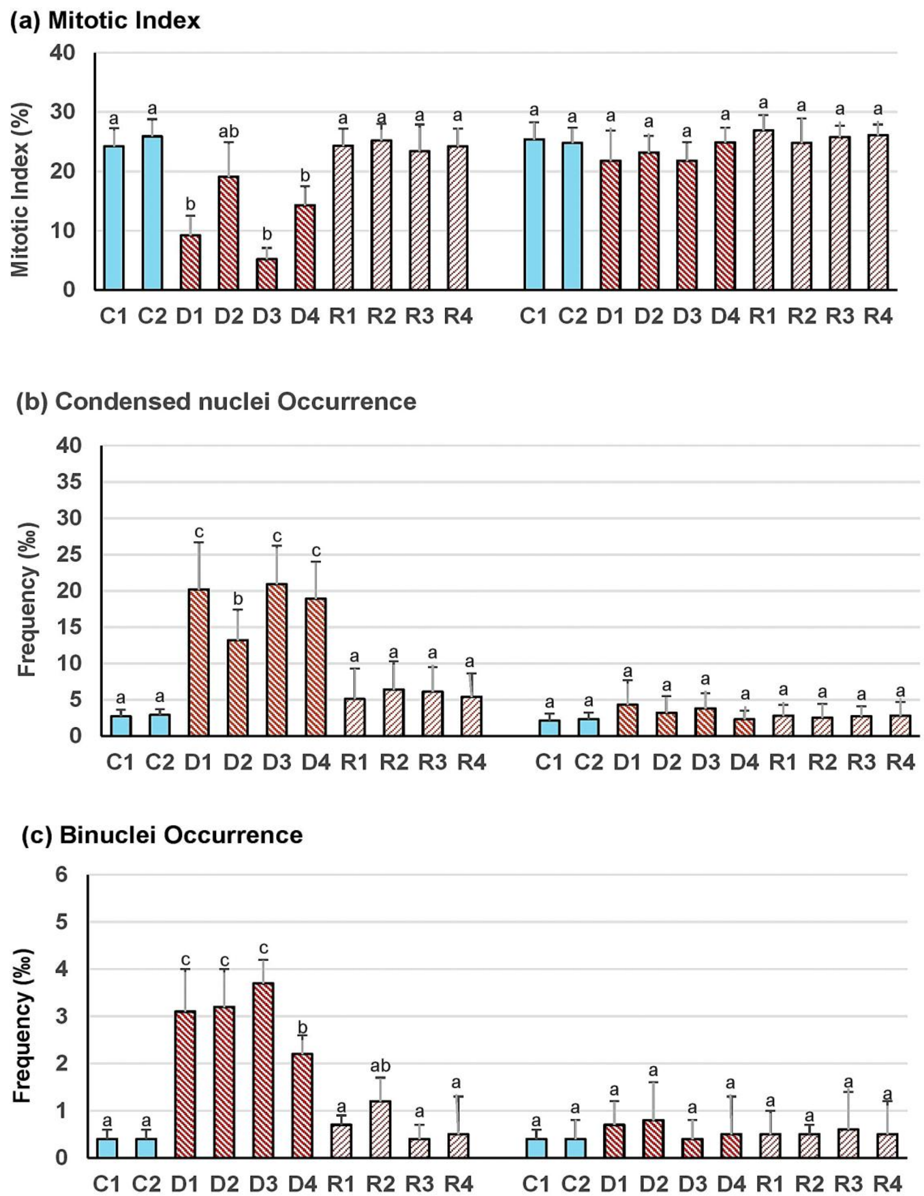

Fig 6. (a) Mitotic index, (b) frequency of cells with condensed nuclei and (c) frequency of binucleated cells in root meristems of *Allium cepa* following 48 hour exposure to potable drinking water from a natural spring (C1), aged tap water (C2) and untreated drinking water samples from selected dug wells used by households with CKDu cases (D1, D2, D3 and D4) and dug wells used by households with no CKDu cases in the same endemic area (R1, R2, R3 and R4). Data are presented as mean ± SEM (n=5). For a specific sampling period, the bars indicated by different letters are significantly different from each other (ANOVA, Tukey's test, $P \leq 0.05$).

Histological structure of the kidneys of fish exposed to dug well water

In freshwater teleosts such as *Cyprinus carpio*, the posterior kidney serves as the primary organ responsible for the excretion of the substantial amounts of water that diffuse into the body across the gill surfaces. Histopathological analysis of the posterior kidney of the fish model, *C. carpio,* was conducted to evaluate potential nephrotoxic effects of untreated dug well water during the dry period, in comparison with fish exposed to control water. The kidneys of fish exposed to control water exhibited normal structure with respect to the Bowman's capsule, glomerulus and proximal and distal segments of renal tubules (Fig. 7a, b). Fish exposed to the untreated dug well water, even for a shorter period (5 days), showed prominent pycnotic nuclei in the epithelium of both proximal and distal segments of the renal tubules (Fig. 7c, d) indicating a cell death process leading to tubular degeneration in all cases. In addition, some fish exposed to water from dug wells used by CKDu cases exhibited an increased Bowman's capsular space in the kidney tissue, which may have resulted from glomerular shrinkage. Histopathological Indices of posterior kidneys of the fish exposed to untreated dug well water during the dry period were significantly higher than those observed in fish exposed to potable drinking water from a natural spring or aged tap water (Fig. 8). In general, higher histopathological indices were observed in the kidneys of fish exposed to dug well water used by CKDu households. Results indicate potential nephrotoxic effects of the untreated dug well water in the CKDu endemic area in the dry period. The observed nephrotoxicity may be attributable to cytogenotoxic effects of the contaminant mixtures present in the dug well water on the kidney tissues of the exposed fish.

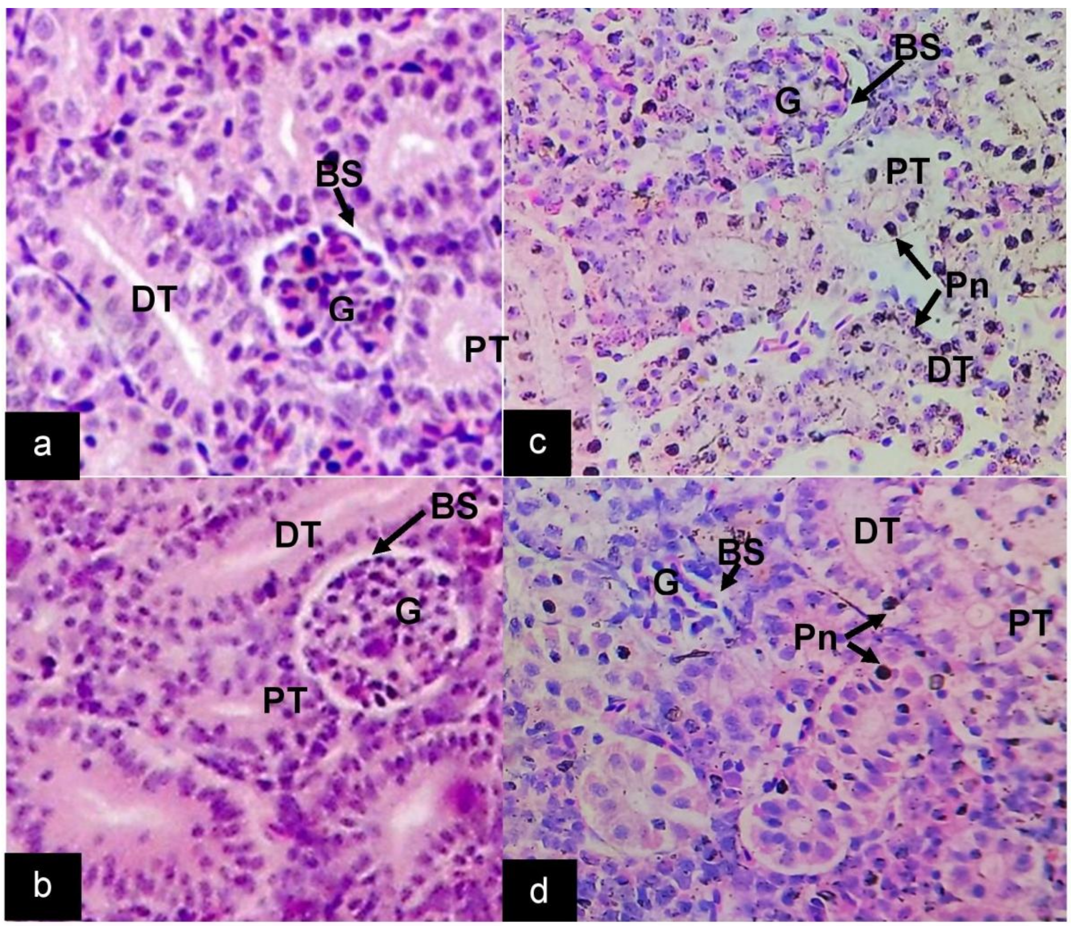

Fig 7. Posterior kidney tissue of *Cyprinus carpio* following short term (5 days) exposure to (a) potable drinking water from a natural spring and (b) aged tap water showing normal histological structure. Posterior kidney tissue of the fish exposed to (c) untreated drinking water from a dug well used by CKDu cases and (d) untreated drinking water from a dug well used by no CKDu cases in the same endemic area showing pycnotic nuclei in the tubular epithelium (Pn). BS: Bowman's capsule space, G:Glomerulus, PT: Proximal convoluted segment of the kidney tubule, DT: Distal convoluted segment of the kidney tubule. (Stained with Hematoxylin and Eosin)

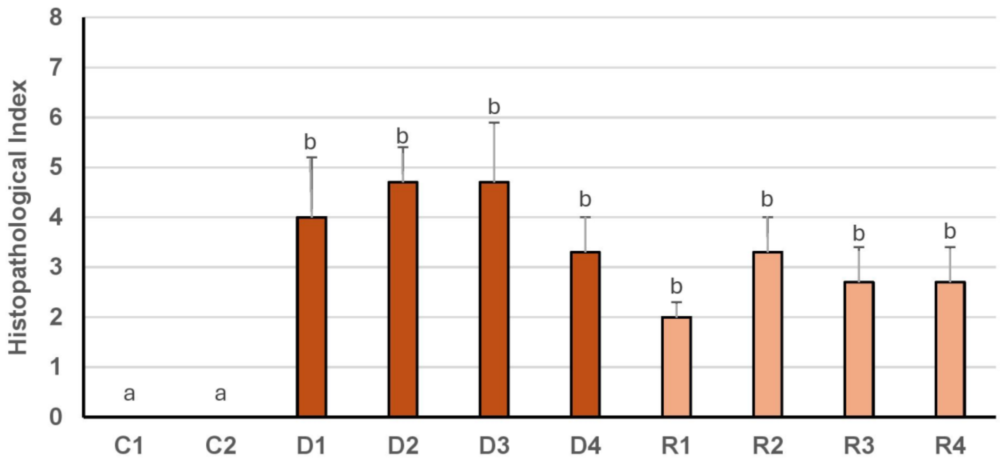

Fig 8. Histopathological Index of posterior kidneys of *Cyprinus carpio* following short term (5 days) exposure to potable drinking water from a natural spring (C1), aged tap water (C2) and untreated drinking water samples from selected dug wells used by households with CKDu cases (D1, D2, D3 and D4) and dug wells used by households with no CKDu cases in the same endemic area (R1, R2, R3 and R4) in the dry period. Data are presented as mean ± SEM (n=3). The bars indicated by different letters are significantly different from each other (ANOVA, Tukey's test, P ≤ 0.05).

Potential links of contaminants in dug well water with cytogenotoxic effects

This study has certain limitations, including the use of judgmental sampling for the selection of dug wells, which may introduce selection bias; small sample sizes in the bioassays (n = 5 fish or onion bulbs per treatment), which may reduce statistical power; and the short exposure period in the fish bioassays (5 days), which does not allow prediction of potential chronic effects. Nevertheless, the overall results of the two *in vivo* cytogenetic bioassays revealed cytotoxic and genotoxic effects of untreated dug well drinking water, particularly from wells used by CKDu households during the dry period. Even though heavy metal(loid)s such as cadmium, arsenic, lead, and mercury are known to cause cytogenotoxic effects, contribution of such metal(loid)s alone for eliciting cytogenotoxicity is highly unlikely as they were not detected in the dug well water used by both groups of households (with one exception of only 2 µg/L arsenic in one dug well). However, electrical conductivity, alkalinity and total hardness levels in the dry period and fluoride levels in both periods were significantly higher in well water used by households with CKDu cases compared to those in well water used by households with no CKDu cases in the same endemic area. Therefore, the observed cytogenotoxic effects may result from the interactive toxicity of the contaminant mixtures, especially fluoride in dug well waters under high ionicity conditions associated with calcium and magnesium hardness. In addition, unknown organic and inorganic contaminants in well water may have contributed to the cytogenotoxic effects, depending on the season. *In vivo* and *in vitro* experimental exposure studies of fish and mammals indicate that fluoride can induce significant genotoxic effects (DeyBhowmik & Chattopadhyay, 2019). Cytogenotoxicity associated with 6.8 mg/L fluoride (as NaF in water) was reported recently in a laboratory exposure study with zebrafish (*Danio rerio*), based on development of erythrocytic nuclear abnormalities and apoptosis of hepatocytes (Mondal et al., 2023). Chronic exposure of common carp to relatively high concentrations of fluoride (40-160 mg/L as NaF) caused oxidative stress and renal apoptosis, resulting in structural kidney damage (Chen et al., 2015). Moreover, destruction of the brush borders in the tubules of the kidneys of adult zebrafish has been reported following chronic exposure to simulated high levels of hardness (800 mg/L as $CaCO_3$) and fluoride (10 mg/L) in water compared to the control group exposed to standard fish culture water (Jia et al., 2024). Short-term exposure of zebrafish larvae to simulated high-hardness and fluoride water also induced kidney damage (Yang et al., 2022). In our study, histopathological assessment of kidney tissues showed that short-term experimental exposure of common carp to the untreated water of dug wells in the CKDu endemic area induced degeneration of renal tubules in all cases and shrinkage of glomeruli in some cases. Further, the estimated 'histopathological index' of kidneys of the fish exposed to dug well water was significantly higher compared to the controls exposed to potable drinking water from other sources, indicating nephrotoxic effects of contaminants present in the dug well water in the CKDu endemic area. Interactive cytogenotoxicity of the mixture of contaminants, especially fluoride, under high ionicity conditions associated with calcium and magnesium hardness, may have induced nephrotoxic effects in the fish exposed to untreated dug well water which warrants further investigations. A review exploring the mechanism of nephrotoxicity of fluoride signifies that accumulation of excessive fluoride in the kidneys can induce apoptosis of cells, leading to renal tissue damage (Wu et al., 2022). Genotoxic damage associated with oxidative stress may play a significant role in the progression of kidney damage (Yüzbaşıoğlu et al., 2024).

A possible nephrotoxic role of exposure to environmental fluoride in CKDu progression has been suggested by Fernando et al. (2020) based on clinical findings of significantly higher urine and serum fluoride levels in CKDu patients compared to control groups in the endemic area of Sri Lanka. Further, Nanayakkara et al. (2020) demonstrated that elevated serum fluoride concentrations in CKDu patients were inversely proportional to their estimated glomerular filtration levels, indicating that fluoride accumulation in the body may further aggravate kidney tissue damage. As poor rural communities in CKDu endemic areas are exposed to the sun for prolonged periods due to farming activities, there is a possibility for consumption of large quantities of untreated dug well water with high fluoride and total hardness levels. When such untreated dug well water is consumed in large quantities for a prolong period, the interactions of fluoride ions with magnesium and calcium ions as well as other unknown organic and inorganic contaminants in water may have an impact on the normal physiological functions of the consumers aggravating the kidney damage. In addition, there can be confounding factors such as exposure to agrochemicals and occupational hazards, which could influence observed effects on the groundwater consumers. Since CKDu is a slowly progressing disease, it is not certain whether households with no CKDu cases in the endemic area will be affected by the disease in the future with prolonged consumption of untreated dug well water. Nevertheless, recent reports indicate that in the North Central Province of Sri Lanka, the incidence of CKDu cases is decreasing, possibly due to the provision of filtered drinking water from reverse osmosis plants as a community service to some areas (Ranasinghe et al., 2024). More scientific evidence is being accumulated to support the hypothesis that chronic dietary exposure to fluoride and other contaminants in untreated groundwater from CKDu prevalent areas constitutes a risk factor for CKDu. Further biological effect-based *in vivo* experiments with animal models under chronic exposure conditions are recommended using untreated groundwater in the CKDu endemic areas and simulated water (with mixtures of suspected contaminants) to clarify the potential association between groundwater dietary exposure and CKDu progression. As the use of bioassays with non-mammalian species provides only a preliminary hazard screening, chronic exposure studies using mammalian models would be more appropriate, given that toxicokinetic and toxicodynamic processes in such models are largely comparable to those in humans.

CONCLUSIONS

In conclusion, the two *in vivo* cytogenetic bioassays revealed cytotoxicity and genotoxicity of untreated dug well water in the CKDu-endemic area, particularly from dug wells used by CKDu-affected households for drinking purposes. Moreover, compared to fish exposed to potable drinking water, fish exposed to the untreated dug well water in the CKDu endemic area induced nephrotoxic effects, which may be associated with cytogenotoxicity of dug well water. Untreated water from such dug wells may pose cytogenotoxic risks to the consumers, influencing CKDu progression. Contribution of cadmium, arsenic, lead and mercury alone in water for eliciting such toxicity is highly unlikely, as they were not detected in the well water in most cases. Yet, electrical conductivity, fluoride and total hardness levels in water of the wells used by households with CKDu cases were significantly higher than those used by non-CKDu households in the same area, especially in the dry period. Observed cytogenotoxic and nephrotoxic effects may be associated with the interaction of fluoride and other unidentified toxicants in dug well waters under high ionicity conditions, mainly associated with water hardness, depending on the season. Further, *in vivo* bioassays, particularly using mammalian models under chronic exposure conditions, are recommended with untreated groundwater used by CKDu-affected communities, alongside simulated water containing mixtures of suspected contaminants, to clarify the cause–effect relationship between consuming untreated groundwater and CKDu progression.

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