Ingestion of dug well water from an area with high prevalence of chronic kidney disease of unknown etiology (CKDu) and development of kidney and liver lesions in rats

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Abstract

Introduction Chronic kidney disease of unknown aetiology (CKDu) is prevalent in the North Central Province (NCP) of Sri Lanka and ingestion of dug well water is considered a potential causative factor. Three CKDu prevalent villages were selected from the NCP based on the number of CKDu patients in the locality.

Methods Forty Wistar rats were divided into four groups with 10 rats each. Group No 1, 2 and 3 were given water from selected dug wells. Control group was given tap water from Colombo. Water samples were analysed for fluoride, iron, arsenic, cadmium and calcium. Histopathological examination of liver and kidney tissues were performed.

Results Significant reduction of glomerular filtration rate (GFR) was observed in two test groups compared to the control group ($p \leq 0.05$). In one group hepatocellular carcinoma with elevated serum liver enzymes was observed whilst hepatitis was observed in another test group ($p<0.05$). But mixed lesions were common in all affected rats. Significantly high renal tubular lesion index was observed in all three experimental groups ($p<0.05$) and high glomerular lesion index ($p=0.017$) was observed in one test group. Cadmium, arsenic and iron contents were below detectable levels in the NCP water sources and tap water from Colombo.

Conclusions Different wells may have different concentrations of environmental toxins and depending on the severity of the toxin contents GFR and grade and type of liver and kidney lesions may vary. High fluoride and other undetected toxins in shallow dug wells may be the causative factors for renal and liver lesions in these Wistar rats.

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Introduction

Chronic kidney disease (CKD) is a public health problem in the North Central Province (NCP) of Sri Lanka [1]. Approximately 75% of the Sri Lankan population live in rural environments and obtain their drinking water directly from the ground. Most of these wells have fluoride concentration of 0.7 mg/l or more and this is considered sufficient to damage kidneys, particularly when there is continuous ingestion of fluoride [1]. The mean fluoride concentration of ground water is 2.3 ppm in the NCP [2]. According to the geographical distribution of CKDu and evidence obtained from histopathological investigations of affected kidneys, the disease is believed to be caused by environmental factors [3]. High fluoride content is common in CKDu affected and non-affected areas in the region. But Ca-bicarbonate type water is more common in the affected region [3]. There are variations in the Na/Ca ratios in the affected and non-affected regions [3]. In addition to the geochemicals in the water, pesticides and heavy metals (cadmium, arsenic, lead and uranium) in soil and water may contribute to the high prevalence of CKD [4]. This animal experiment was designed to investigate the direct relationship between the quality of consumed water and development of renal lesions.

Methods

Forty, six-week old Wistar rats (190±10g) bred and maintained at the Medical Research Institute (MRI), Colombo were randomly divided into four groups of five males and five females. The required sample size was six rats for each group, but 10 rats were recruited in order to avoid a cluster effect. The animals were maintained under standard conditions and fed with a diet prepared according to a WHO formula using locally available ingredients [5].

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Their body weights and other relevant baseline biochemical parameters were analysed. Three dug wells numbered 1-3 were selected for the experiment. The dug well number 1 was selected from New Town Medirigiriya (NTM), where five CKD patients were detected in one family, two had died while others were surviving. They have consumed water from that well for more than 30 years. Water source number 2 was selected from Bisobandaragama (BB) in the Mediririgiriya division. It was a community dug well shared by three families. They have been using water from that well for more than 11 years. Two families had at least one CKD patient. Water source number 3 was selected from Divuldamana (DD) from Dimbulagala division. Three families have been consuming water from this source for more than six years and no kidney patients had been detected so far.

Water was collected into 20 liter plastic containers which were first washed with tap water and then distilled water. The containers were labeled and transported to MRI at monthly intervals and stored in a cold room (2°C- 8°C) until use. Animals in Group 1, 2 and 3 were given water collected from New Town Medirigiya (NTM), Bisobandaragama (BB) in Medirigiriya division and Divuldamana (DD) in Dimbulagala division respectively. Animals in Group 4 received tap water from Colombo and remained as the control. Every week, the animals were weighed and the feed and water consumption were recorded.

Voided urine was collected into a clean tray and 500µl were aspirated into a sterile micro tube using a sterile pipette. The samples were analysed using dipstick method for ten parameters at weekly intervals (Plamatec Laboratory Products Limited, UK). Urine samples were stored at -20°C for further investigation. Animals were anaesthetised using anaesthetic ether, they were placed in rat holders, the tail was dipped in lukewarm water (40°C) for 1-2 min and 0.5-1 ml of blood was collected from the lateral tail vein using a 23-guage needle attached to a 1 ml syringe. Serum was separated by centrifugation of blood at 10,000 rpm for 5 minutes and stored at -20°C [6].

Animals were placed in metabolic cages with urine faecal separation device at monthly intervals and their 24 hour urine samples were collected and measured. Glomerular filtration rate was calculated at regular intervals. Freshly voided urine samples were collected from rats assigned to metabolic cages. Urinary β_2 microglobulin levels were measured by the solid-phase chemiluminescent immunometric assay (Immulite/ immulite 1000 Beta-2 Microglobulin PILKBM-112006-12-29).

Serum electrophoresis was performed using an electrophoretic chamber (Bender and Hobein). Cellulose acetate paper was soaked in barbiturate buffer. Serum samples (50µl) were placed in each well of the electrophoretic chamber and human serum was used as a control. All samples were transferred to cellulose acetate paper using the sample applicator and placed in the electrophoretic chamber and a constant current was delivered. Cellulose acetate paper was stained with Amido black stain and after 1 minute, it was washed with acetic acid and electrophoretic mobility was observed [7].

Urine samples were concentrated overnight using saturated sugar solution [7]. The following day serum and its corresponding urine samples were placed in cellulose acetate paper using the method described previously. After the experimental period of nine months, serum analysis was performed using C-CYS quantitative turbid metric test using Cystatin C-turbilatex test kits (Spinreact Cystatin C-turbilatex). Urine samples were assessed using human creatinine liquicolor photometric colorimetric test for kinetic measurement, using semi micro method, at monthly intervals using the instructions given by the manufacturer [8]. Serum creatinine levels were assessed at monthly intervals using creatinine liquicolor photometric colorimetric test by human reagent kits according to the instructions given by the manufacturer [8]. GFR was measured by using the following formula; (Urinary creatinine × 24 hour urine output) / (Serum creatinine × 1440).

GFR values were calculated and divided by the body weight in kg to obtain the glomerular filtration rate of rats [9].

After 15 months, animals were humanely euthanized. Post-mortem examination was performed. Body weights were recorded. Tissue samples for histopathology were collected into 10% phosphate buffered formalin. The tissues were fixed for 24-48 hours and tissue sections were processed using a standard method [10].

Slides were deparaffinised and deionised before placing in 40 ml of periodic acid solution contained in a plastic jar and microwaved at 800 watts for 10 seconds. They were then rinsed well by changing the deionised water several times [11]. Activity index was graded using periportal or periseptal interface hepatitis, confluent necrosis, focal (spotty) lytic necrosis, apoptosis, focal inflammation and portal inflammation. Maximum possible score for activity index was 18. For chronicity index, different levels of fibrosis were graded. Maximum possible score was 6 [12].

The severity of kidney lesions was assessed by counting the severity of the lesions in 30 randomly selected glomeruli. Partial to complete sclerosis of glomeruli and glomerular basement membrane thickening was identified as main lesions. The severity of the lesions were graded from 0 to 4 points according to the percentage of morphological changes on each glomerulus and assigned a score (0=0%, 1^+=1-25%, 2^+=26%-50%, 3^+=51-75%, 4^+=76-100%). The number of glomeruli showing; no lesions=n_0, 1^+ severity score=n_1, 2^+ severity score =n_2, 3^+ severity score =n_3 and 4^+ severity score =n_4. Thirty glomeruli were counted and index was obtained by using the following formula; $(0 \times n_0 + 1x\ n_1 + 2\ x\ n_2 + 3\ x\ n_3 + 4\ x\ n_4)/30$ [13].

Morphological analysis was done on 10 microscopic fields per section examined at a magnification of × 200 and data were averaged.

TI lesion score was assessed as follows. Mild, moderate and severe tubular lesions were observed and graded from 1-4 points according to the severity of the lesions. Different grade of peritubular and periglomerular lesions were identified and graded (0= 0%, 1^+= 1%-25%, 2^+= 26%-50%, 3^+= 51%-75% and 4^+= 76%-100%) [14].

Water cadmium levels were assayed using standard APHA 3113B, arsenic contents were analysed using APHA 3114 C method and calcium levels were assayed using APHA 3500 Ca-D. Sodium levels were analysed using APHA 3111B, total iron was assayed using APHA 3111B method or Direct Air-Acetylene Flame Method and fluoride level of water was analysed using APHA 4500-PC method [15].

Ethics approval for this study was obtained from the Ethics Review Committee of the Medical Research Institute, Colombo.

Wilcoxon sign rank was used for non parametric values and paired t test for parametric data.

Results

The baseline values were GFR1.0±.58 ml/min/kg, serum creatinine 0.74±0.33 mg/dl, ALT33.3±10.37U/l and AST171.07U/l. No differences observed in urine analysis using dipstick method throughout the experimental period. Urinary β_2 globulin was <4 ng/ml. Urine electrophoresis did not produce a characteristic band of β_2 globulin. There was no difference in serum cystatin C after nine months of experimental period (p>0.05).

In the experimental groups, the GFR was lower than the control group throughout the experimental period. However compared to the control group, a significantly high GFR was observed in rats maintained with water from Medirigiriya after four months of the experiment. After six months, rats who ingested water from Medirigiriya and Dimbulagala had significantly lower GFR compared to the control (p≤0.05) whilst rats who ingested water from Bisobandaragama had significant reduction in GFR after nine months which was persistent (p≤0.05). No significant difference was observed in serum creatinine levels in test groups (p>0.05). No significant difference observed in serum cystatin C levels (p>0.05) after 9 months of the experimental period.

One rat who ingested water from Medirigiriya died. Four rats developed hepatocellular carcinoma while one rat developed mild hepatitis. Mild to moderate steatosis was prominent in all liver tissues and sinusoidal dilation was observed in seven rats. One rat who ingested water from Divuldamana died. Focal adenoma formation was observed in one rat while hepatitis was observed in two rats. Hepatocellular carcinoma was observed in one rat. Steatosis and sinusoidal dilatation were observed in most of the rats.

One rat that ingested water from Bisobandaragama died and five rats developed moderate to chronic hepatitis. One rat developed hepatocellular carcinoma and another had portal fibrosis. Steatosis and sinusoidal dilation were observed in most of the rats. None of the rats that ingested tap water from Colombo died. Focal nodular hyperplasia was observed in three rats and mild bile duct hyperplasia in one rat. Mild to moderate steatosis was observed in most of the rats while in four rats sinusoidal dilation was observed.

Rats that ingested water from Medirigiriya had high prevalence of hepatocellular carcinoma (p=0.0177) whilst rats that ingested water from Bisobandaragama had significantly high incidence of hepatitis (p=0.0018). Sinusoidal dilation was a prominent feature in the experimental groups and it was statistically significant in rats who ingested water from Medirigiriya (p=0.0373) and Divuldamana (p=0.0094). Hyperchromatic nuclei was a prominent feature in the experimental groups and it was statistically significant in rats who ingested water from Medirigiriya (p=0.0106) where a significantly high number of hepatocellular carcinoma was also observed.

Interstitial nephritis was observed in eight rats who ingested water from Medirigiriya. To assess the glomerular lesions, 30 glomeruli were observed in each rat and mild sclerosis was observed in eight rats. The next most prominent glomerular lesion was basement membrane thickening. Seven rats who ingested water from Divuldamana developed mild to severe interstitial nephritis while glomerular lesions were observed in eight rats. Among the rats that ingested water from Bisobandaragama severe interstitial nephritis was observed in three rats while glomerular sclerosis was observed in four rats. In the control group, mild peritubular inflammatory cell infiltrates were observed in two rats and mild sclerosis was observed in three rats. In all three experimental groups significantly high tubular lesion index was observed (p<0.05). Glomerular lesion index was statistically significant in animals that ingested dug well water from Divuldamana (p=0.017). There was a significant association between the consumption of water from selected dug wells and development of chronic interstitial nephritis in Wistar rats.

Mineral and heavy metal composition were analysed at the Industrial Institute of Technology of Sri Lanka. Cadmium, arsenic and iron levels were below detectable levels in selected dug wells and tap water from Colombo. Calcium and fluoride content in selected dug wells were higher than in pipe water from Colombo (Table 1).

Table 1. Mineral and heavy metal composition of selected dug wells and tap water from Colombo (Tested by Industrial Technology Institute of Sri Lanka)

Test	*Medirigiriya*	*Bisobandaragama*	*Divuldamana*	*Tap water from Colombo*
Fluoride (mg/dl)as fluoride	1.3 mg/dl	0.57 mg/dl	0.48 mg/dl	Below detectable level
Total iron (mg/L)	Below detectable level	Below detectable level	Below detectable level	Below detectable level
Calcium (mg/L)	46 mg/L	40 mg/L	21 mg/L	6mg/L
Cadmium (mg/L)(0.001mg/L)	Below detectable level	Below detectable level	Below detectable level	Below detectable level
Arsenic (mg/L)(0.001mg/L)	Below detectable level	Below detectable level	Below detectable level	Below detectable level

Discussion

Kidney plays an important role in body homeostasis and CKD can affect every body system [16]. In our study significantly low GFR was observed in rats who ingested water from high disease prevalent areas compared to tap water from Colombo. Histopathology showed that there were significantly higher tubular lesion index in all three experimental groups. Rats that ingested water from Divuldamana had significantly high tubular lesion index together with significantly high glomerular lesion index. Though it is not significant, mixed lesions of glomerular sclerosis and basement membrane thickening were observed in all the test groups. Most prominent kidney lesions were mild to moderate focal chronic inflammatory cell infiltrates in the renal interstitium. Interstitial fibrosis and tubular atrophy with or without nonspecific interstitial mononuclear cell infiltration are the most prominent lesion in human patients [17].

Rats who ingested water from Medirigiriya had a higher incidence of hepatocellular carcinoma whereas rats who ingested water from Bisobandaragama had a higher incidence of hepatitis. Liver is the main organ involved in detoxification. Therefore, severity of liver lesions can vary according to the concentrations of toxins in the water.

Humans differ from animals with regards to their isoform composition, expression and catalytic activities. Cytochrome P450 enzymes (CYPs) are one of the main enzymes involved in various oxidative reactions and metabolism. CYPE1 shows no significant difference between species and therefore extrapolation of findings between species is possible. Species specific isoforms such as CYP1A, CYP2C, CYP2D and CYP3A have significant intra species differences in metabolism. Body size and liver weight as a percentage of body weight is important in metabolism. Hepatic enzyme such as Cytochrome P450/gr body weight is higher in small animals than in humans. This suggests small animals tend to eliminate metabolites rapidly than humans [18]. In rodents hepatic CYP isoform induction can be associated with the formation of tumours in liver, thyroid and other tissues [19]. This may be the reason for hepatic lesions in Wistar rats which are not observed in humans living in CKDu affected villages. We found that content of heavy metals such as arsenic and cadmium were below detectable levels in the well water from affected areas.

We conclude that rats who ingested water from high disease prevalent villages were susceptible to interstitial nephritis. Ingestion of fluoride alone or in combination with some other unmeasured component in water may be responsible for renal and hepatic changes in Wistar rats.

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Conflicts of interest

All authors declare that there are no conflicts of interest.

References

1. Dissanayake CB. Water quality in the dry zone of Sri Lanka. Some interesting Health Aspects. *Journal of National Science Foundation of Sri Lanka* 2005; **33**: 161-8.
2. Herath KRPK, Illeperuma O, Darmawardena HH, Kenneth J, Haller. *Environmental Health Risk for the Chronic Renal Failure in Sri Lanka*. 31st Congress on Science and Technology of Thailand at Suranaree University of Technology October 2005; 18-20.
3. Chandrajith R, Dissanayake CB, Ariyarathna T, Herath HM, Padmasiri JP. Dose dependent Na and Ca in fluoride-rich drinking water – Another major cause of chronic Renal Failure in tropical regions. *Science of the Total Environment* 2011; **409**: 671-5.
4. Chronic kidney disease of unknown etiology (CKDu). A new threat to health http://slwater.iwmi.org/sites/default/files/DocumentRoot/1029.pdf

5. Saboudry MA. *Breeding and care of Laboratory Animals*. Geneva: World Health Organization, Health Laboratory Technology Unit, 1988.

6. Waynforth HB, Flecknell PA. *Experimental and Surgical Techniques in the Rat.* London: Academic Press Limited, 1992.

7. Medical Research Institute. *Manual of Standard Operation Procedure*. Colombo: Department of Immunology, Medical Research Institute, Colombo, 2009.

8. Thammitiyagodage MG. *Identification of possible causative factors in Chronic Kidney Disease (CKD) in North Central Province (NCP) by an animal Experimentation*, Mphil Thesis, University of Peradeniya, Sri Lanka 2012; 32-3.

9. Aiello AE, Mays A et al. Non Infectious diseases of urinary system in small Animals. In: Khan CM, Line S. *The Merck Veterinary Manual* 8th ed. New Jersey: Whitehouse Station, N.J.: Merck & Co.1998: 1134-6.

10. Alam MI, Ian W. *The haemotoxylin and eosin. In theory and practice of histological techniques*. John DB and Alan Seds, Wellington: Whitehall Books Ltd, 1996.

11. John DB, Alan S. Theory and Practice of Histological Techniques, 4th Edition, Edinburgh: Churchill and Livingstone, 1996.

12. Rosai J, Ackerman LV, *Surgical Pathology*, 9th ed. Milan: Mosby 2004.

13. Schaier M, Lehrke I, Schade K, et al. Isotretinoin alleviate renal damage in rat chronic Glomerulonephritis. *Kidney Int* 2001; **60**: 2222- 34.

14. Toblli JE, Ferder L, Angerosa M, Inserra F. Effect of Amlodipine on tubulointerstitial lesions in normotensive Hyperoxaluric rats. *Hypertension* 1999; **34**: 854-8.

15. Examination of Water and Waste water: Inorganic non metals (4000) https://archive.org/stream/gov.law.apha.method. 4500-si.1992/apha.method.4500-si.1992_djvu.txt

16. Snivelly CS, Gutierrez C. Chronic kidney disease: prevention treatment of common complications. *Am Fam Physician* 2004; **70**: 1921-8.

17. Nanayakkara S, Komiya T, Ratnathunga N, et al. Tubular interstitial damage as the major pathologic lesion in endemic chronic disease among farmers in North Central Province of Sri Lanka. *Environ Health Prev Med* 2012; **17**: 213-21.

18. Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug MetabToxicol* 2006; **6**: 875-94.

19. Graham MJ, Lake BG. Induction of drug metabolism: Species differences and toxicological relevance. *Toxicology* 2008; **254**: 184-91.