Changes In Immunological Response In Wistar Rats That Ingested Water From High And Low Disease Prevalent Areas From The North Central Province (NCP) And Low Disease Prevalent Colombo, Sri Lanka And Their Co-Relations To Histopathological Changes.

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Abstract

Background

Chronic Kidney Disease of unknown etiology (CKDu) is prevalent in North Central Province (NCP) of Sri Lanka and ingestion of ground water is identified as one of the causative factors. Majority of the population in the NCP consume un-boiled dug well water. Objective of this study was to find out the haematological and immunological variations in Wistar rats that ingested water from high and low disease prevalent areas from the NCP and low disease prevalent Colombo and correlated the findings with histopathological changes.

Method

Wistar rats (60) were recruited to the study and their baseline WBC, DC, CD4+, CD8+, serum cytokines, creatinine, ALT, AST and BUN levels were measured. Rats were randomly divided into 6 groups by assigning 10 rats into each group. Groups 1, 2 and 3 were given water from high disease prevalent New Town Medirigiriya (NTM), Bisobandaragama (BB) and Divuldamana (DD) and group 4 was given boiled water from NTM (NTMB). Group 5 and 6 were given water from low diseases prevalent Huruluwewa (HW) from NCP and low disease prevalent Colombo (CO). Serum cytokines (IL1ß, IL6, TNFα) were measured after 8 months and other parameters and tissue cytokines were measured after 14 months. Histopathology was performed in kidney and liver tissues.

Results

Serum TNFα levels were significantly elevated in rats from DD and BB but tissue TNFα levels were significantly elevated only in rats from DD. Rats from high diseases prevalent areas had significantly high CD4+ and CD8+ cell than those from low disease prevalent HW and CO. Immunological findings were correlated with the changes observed in the histopathology. There was a co-relation between the kidney Tubular Interstitial Lesion index and liver lesions.

Conclusion

TNFα and CD4+ and CD8+ lymphocyte had an impact on kidney damage. Rats with severe TI lesions reported high percentage of portal tracts and parenchymal lesions in the liver and this expression was minimum in CO. Boiled water can reduce the liver damage but not been able to significantly reduce the kidney damage. Immune therapy targeting the CD4+, CD8+ and TNFα may reduce the disease burden in the early stage.

Background

Approximately two hundred million people are diagnosed with chronic kidney disease (CKD) annually worldwide with the majority from low to middle income countries in Asia and sub-Saharan Africa [1].
stage renal disease (ESRD) resulting from chronic kidney disease requires costly renal replacement therapy in the form of renal transplantation or dialysis [2]. As such, it has become an economic burden for developing countries.

CKD is defined as kidney damage or reduction in glomerular filtration rate < 60 mL/min/1.73 m² for 3 months or more, irrespective of the underlying causes for such changes [3]. Diabetic nephropathy is the main cause of renal impairment in developing countries. In addition, multiple risk factors such as hypertension, hyperlipidemia and smoking have also been identified. Infectious diseases which can lead to glomerulonephritis are also considered as important contributory factors for CKD in developing countries [4]. A new disease entity, chronic kidney disease of unknown etiology (CKDu) was identified among Sri Lankan farming communities in the 1990s, where an obvious cause for CKD, such as hypertension or diabetes, could not be identified [5]. It is a major public health problem in the North Central Province (NCP) of Sri Lanka and the districts of Anuradhapura and Polonnaruwa are the most affected. The NCP is the area with the highest CKD burden in the country with 10% of the adult population being affected, of which, 27%, have been diagnosed with CKDu [6]. Patients with CKDu remain asymptomatic for a prolonged period [7]. Albuminuria is one of the common manifestations in many forms of CKD. It is defined as the presence of more than 30 mg/g, albumin: creatinine ratio in two or three spot urine samples [4]. A retrospective study of 211 renal biopsies obtained from asymptomatic individuals with albuminuria, living in CKDu endemic regions of the NCP for more than five years (2007–2011), were subjected to histopathological analysis. Patients with hypertension, diabetes and histological diagnosis of primary kidney disease, immune complex mediated diseases and other renal disease secondary to systemic diseases were excluded from the study population [8]. The renal biopsies were categorized into 6 groups, ranging from no detectable histopathological changes (category 0), mild interstitial inflammation and fibrosis, tubular atrophy without glomerular sclerosis (category 1) to various grades depending on the severity of the lesions. Category 6 included patients with severely impaired kidneys, including severe interstitial fibrosis, tubular atrophy and interstitial inflammation of any degree. Results revealed that 88.6% of the study population belonged to groups 1, 2 and 3, having interstitial fibrosis, interstitial inflammation and tubular atrophy of varying degrees as the prominent pathological lesions. The vast majority of the samples were diagnosed with interstitial fibrosis as the main histopathological finding. Of the samples diagnosed with interstitial fibrosis, 42.2% did not have any inflammation. All patients with interstitial inflammation had interstitial fibrosis (Category 4). Of the patients in category 1, 83.7% had interstitial fibrosis without interstitial inflammation or tubular atrophy. As such, interstitial fibrosis is identified as the earliest pathological feature in asymptomatic individuals. A retrospective study by Wijetunga et al evaluated 251 renal biopsies from individuals identified to have a primary interstitial kidney disease from CKDu endemic areas. The 251 patients were divided into five categories based on their glomerular filtration rate (GFR). Patients in stages 1, II, III, IV and V had GFR > 90, 60–89, 30–59,15–29 and < 15 mL/min respectively. Of the patients in stage 1 and 11, the prominent pathological feature was interstitial fibrosis. Of the patients in stage 1, 72.2% did not have any inflammation whilst prominent concomitant interstitial inflammation was also observed in those in stages 111 and IV. It is apparent that the initial disease is a sclerosing type of interstitial disease with no or minimal inflammation. Interstitial inflammation observed in more advanced stages of the disease is probably a secondary manifestation [9]. After the establishment of interstitial fibrosis, the disease
progresses resulting in tubular interstitial inflammation and glomerular changes [9]. The authors have offered two hypotheses as explanations for this phenomenon, (a) interstitial fibrosis being healed foci of interstitial inflammation and (b) fibrosis being the initial response to injury [8]).

A previous study conducted by our team using in vivo Wistar rat animal model revealed that long term ingestion of dug well water from high and low disease prevalent areas of the NCP led to peritubular, non-suppurative interstitial nephritis in Wistar rats. The inflammatory cell infiltration was observed in the latter part of the experimental period and the most prominent lesion was peritubular inflammatory infiltrates as observed in CKDu patients in NCP, Sri Lanka [10]. In a subsequent study carried out using the same water sources and the same animal model revealed that, out of the rats that ingested water from different sources, the most severe lesions were observed in those who ingested un boiled and boiled water from New Town Medirigiriya (NTM and NTMB, respectively) and Divuldamana (DD) from the NCP. However, the severity and the distribution of these kidney lesions were different in the two experimental groups. In rats that ingested water from NTM and NTMB, focal, chronic to moderate interstitial inflammation were observed whilst in rats from DD, in addition to the inflammatory lesions, severe tissue destruction and fibrosis were also observed and lesions were equally distributed throughout the kidney [11].

The reason for the renal injury was postulated to be an unidentified toxins/toxin present in dug well water from these areas [11]. Authors hypothesized that potential toxic mediators present in the dug well water can directly damage the kidney tissues.

Usually, cytokines are released from the sites of injury as a response to local inflammation. Following kidney injury, cytokines are released from circulating mononuclear cells, including lymphocytes and / or from the injured kidney cells. Their actions are autocrine, paracrine or even systemic. They may be pro-inflammatory, for example attracting and activating leucocytes to the site of injury, or anti-inflammatory. Variation in the pattern of pro and anti-inflammatory cytokine expression determines the activation and characterization of acute kidney injury (AKI), glomerulonephritis (GN) and end stage kidney disease (ESKD). Plasma levels and gene expression of certain cytokines can have predictive value in kidney disease [12]. As cytokine activity is mainly autocrine or paracrine, they are usually present in body tissues and fluids in very low concentrations, and may even be undetectable in plasma [12]. As such, measurement of plasma cytokines has limited applications. However, CKD is a valid model to describe a cytokine mediated immune reaction as pro inflammatory cytokines are counterbalanced at several levels in the pathogenesis of CKD. This process is complex as most of the cytokines are inhibited by specific cytokine inhibitors. Secretion of IL1ß is linked to secretion of IL- 1 receptor agonist and neutralizes the effect of IL1ß. Similarly, mechanism of TNFα can be neutralized by soluble TNFα receptors [12]. Dysregulated immune system can have a direct or indirect impact on kidney tissues. However, it can also be a result of uncontrolled activation of complement pathways. Though the range of dysregulated immune mediated mechanisms are broad and complex the pathway that can initiate potential renal damage is the same. Loss of renal homeostasis results from recruitment of peripheral cells and damage to renal tissues. Uncontrolled and uncoordinated attempts to repair the damaged kidney after immune mediated or non-immune mediated disease can lead to fibrosis of important renal structures [13].
In the same *in vivo* animal experiments using a Wistar rat model, severe hepatocellular carcinoma and hepatitis, features which are not observed among CKDu affected individuals in NCP, were identified. The authors concluded that the reason for this difference was due to a deficiency in certain species-specific isoform enzymes in the cytochrome P450 enzyme system in the liver and a higher cytochrome P450/gr body weight in small animals compared to humans [10].

The main objective of the present study is to identify the function of the inflammatory cells and expression of pro and anti-inflammatory cytokines in the pathogenesis of chronic kidney disease. The following specific objectives were also included.

1. To determine whether a toxin causes damage leading directly to fibrosis, or via inflammation.
2. To determine whether the damage results in the production of pro and anti-inflammatory cytokines leading to fibrosis/inflammation.
3. To elaborate on how the immune mediated renal response occurs during the pathogenesis of end stage renal disease (ESRD) and how it is reflected in the histopathology in kidney and liver tissues.

**Ethical Clearance**

Ethics clearance was obtained from the Ethics Review Committee of the Medical Research Institute, Colombo, Sri Lanka (Ethics Review Number 11/2012). The animal experiments were performed according to international guidelines of the use and care of laboratory animals in research.

**Methodology**

**Animals**

*Rattus norvegicus*, Wistar rats (origin: Clear, Japan, Inc.) bred and maintained at the MRI under clean microbiological conditions were used for the experiment. They were maintained in an air-conditioned room (22 °C- 24 °C) with relative humidity in the range of 40% -70%, 12-hour light dark cycles and 15-16 air exchange per hour. These rooms were provided with filtered air by the medium efficiency filters. They were maintained on a ration prepared according to World Health Organization (WHO) guideline using locally available feed ingredients at the Medical Research Institute. Rats were maintained in standard polypropylene cages (3-4/cage) and water was provided using transparent water bottles designed for rat cages. All the rats had access to water and feed *ad libitum*. Autoclaved wood shaving was used as their bedding materials. All the animal experiments were carried out by a qualified and experienced veterinary surgeon.

**Sample size calculation**

Sample calculation was done as described by Charan *et. al*[14] in the study conducted by Thammitiyagodage *et al* in 2020 [10].
Animal Experiment

Sex balanced (1:1) 12 weeks old male/female Wistar rats (60) were recruited to the study. Males with a body weight range of 240 g ± 8 and females with a body weight range of 183.6 g ± 3.6 were randomly divided into 6 groups, with 10 rats in each group. Groups 1, 2, 3, 4 and 5 were the test groups and No 6 was the negative control. Rats in Groups 1-3 were given water collected from high disease prevalent areas of the NCP, namely New Town Medirigiriya (NTM), Bisobandaragama (BB), Divuldamana (DD) whilst group 4 was given boiled water from NTM (NTMB). Group 5 was given water collected from Huruluwewa (HW), a low disease prevalent area of the NCP. Group 6 was given water collected from the low disease prevalent city of Colombo (CO)[12]. HW was considered as the control group for the NCP and CO was considered as negative control.

Blood collection

Blood collection was carried out by the method described by Fleckenell *et al* after mild sedation using gaseous anaesthesia [15]. Blood (1mL) was collected from each rat. Subsequently, 300 µL of blood was placed in an Eppendorf tube coated with ethylene diamine tetra acetic acid (EDTA) and thin blood smears were prepared. An aliquot (0.7 mL) was separately placed in a 1 mL Eppendorf tube and serum was separated after centrifugation at 12,000 rpm for five minutes. Serum was stored at -20°C until further analysis.

Blood samples were collected on three occasions (a) at the beginning of the experiment (baseline), (b) at the completion of 8 months and (c) at the completion of 14 months of the experiment. All samples were subjected to haematological, immunological and biochemical analysis as described below. Blood sample collection and sample analysis were carried out by two different technical expertise to maintain blinding.

White cell (WBC) counts

Blood was diluted in a solution that lyses red blood cells (2% glacial acetic acid) prepared by mixing 2 mL glacial acetic acid in 98 mL distilled water. Concurrently, 5 drops of methylene blue also were incorporated to the same solution. Nucleated cells were mounted in a known volume of prepared suspension and cell counts were estimated using a hemocytometer.

Differential counts

Thin blood smears were made and stained with Leishman stain. Slides were air dried and smears were examined under oil immersion using a light microscope (x1000). Approximately 100 white blood cells were counted in five fields in the stained slide and different types of white blood cells were identified according to the morphology of the cells. Percentage of different cell counts/100 WBC counts were calculated.
Serum Aspartate transaminase (AST) analysis

The assay was performed using commercially available Pointe scientific Inc reagent to detect serum AST. Each serum samples (100 µL) were reacted with 1000 µL of pre-prepared working reagent and the reading was directly obtained using a Stat Fax 3300 semi-automated biochemistry analyzer as per manufacturer's instruction.

Serum Alanine transaminase (ALT) analysis

The assay was performed using commercially available Pointe scientific Inc. reagent to detect serum AST. Each serum samples (100 µL) were reacted with 1000 µL of pre-prepared working reagent and the reading was directly obtained using a Stat Fax 3300 semi-automated biochemistry analyzer as per manufacturer's instruction.

Flowcytometry analysis of CD $4^+$ and CD $8^+$ cell counts

CD $4^+$ and CD $8^+$ cell counts in blood was analyzed using monoclonal antibodies (Rat CD 4 FITC ox-35 and Rat CD 8 FITC ox-8 supplied by R & D Systems) by flowcytometry (Facscaliber- Becton Dickinson).

Detection of serum and hepatic cytokines

TNFα, IL6 and IL1β cytokine levels in serum and liver homogenate were assessed using rat TNFα, IL6 and IL1β Quantikine ELISA kits (R & D Systems) as per manufacturer's instructions. Each sample was performed in triplicate and quantification carried out by referring to the pre-plotted standard curve.

Tissue preparation for histopathology

All experimental animals were humanely euthanized after exposing them to CO₂ and liver and kidney samples prepared for histopathology as previously described by Thammitiyagodage et al [10 &11]. Kidney and liver tissues were examined under light microscopic investigations and prominent pathological lesions were identified. Severity of the kidney lesions were graded according to the scale previously described for rat kidney [10]. Non affected to affected kidney tissue ratios were calculated. Percentage of prominent histological lesions observed in each rat groups also were identified [11].

Liver tissue collection and processing

The left lateral hepatic lobe of each rats was excised, quick frozen in liquid nitrogen and stored at -80°C. Briefly, each liver sample was gently homogenized in chilled phosphate buffered saline (PBS) containing
protease inhibitors, the homogenate was centrifuged and the supernatant was stored at -80\(^\circ\)C until their cytokine levels are assessed.

**Assessment of protein in liver homogenate**

Total proteins in the previously prepared supernatant of the liver homogenate were assessed by using Bradford reagent in triplicate using a pre-plotted standard curve.

**Statistical analysis**

Statistical analysis was performed using the SPSS 16 statistical package. Comparison of all the biochemical and immunological parameters at baseline and two subsequent occasions (at 8 months and 14 months) were analyzed using one-way ANOVA with LSD post hoc. Cytokines levels were analyzed using regression and mean differences were compared using one-way ANOVA.

**Results**

**Effect of drinking water on changes in haematological parameters**

Basic haematological parameters (WBC/ DC) in different study groups at baseline, 8 and 14-months post baseline assessments are shown in Table 1.

Table 1 Changes in basic hematology (Total WBC, Neutrophils and Lymphocytes) at the baseline (generalized outcome n=30), 8- and 14-month post baseline (n=10 per each experimental group) (Mean ± SEM).
<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Assessment parameter</th>
<th>Time of the assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBC</td>
<td>Neutrophils</td>
</tr>
<tr>
<td></td>
<td>15,800±591</td>
<td>18.41±0.86</td>
</tr>
<tr>
<td>NTM</td>
<td>10,360±808</td>
<td>24±1.67</td>
</tr>
<tr>
<td></td>
<td>9,467±217</td>
<td>22±0.87</td>
</tr>
<tr>
<td>NTMB</td>
<td>10,838±843</td>
<td>23.3±0.7</td>
</tr>
<tr>
<td></td>
<td>11,817±1116</td>
<td>32±2.3</td>
</tr>
<tr>
<td>DD</td>
<td>14,670±606*</td>
<td>27±1.27*</td>
</tr>
<tr>
<td></td>
<td>12,133±589</td>
<td>30±1.7</td>
</tr>
<tr>
<td>BB</td>
<td>11,425±787</td>
<td>19±1.75</td>
</tr>
<tr>
<td></td>
<td>10,567±557</td>
<td>29±1.8</td>
</tr>
<tr>
<td>HW</td>
<td>12,427±505</td>
<td>22.6±0.95</td>
</tr>
<tr>
<td></td>
<td>9,967±803</td>
<td>24±1.3</td>
</tr>
<tr>
<td>CO</td>
<td>10,270±705</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>10,466±557</td>
<td>31.5±2.3</td>
</tr>
</tbody>
</table>

Past baseline assessment n=(10) each experimental groups.

When comparing total leucocyte (WBC) counts obtained at 8-months post baseline with the baseline values, all groups had significantly reduced total WBC counts except the rats that ingested water from DD (p<0.05). A significant increase in the neutrophil counts were observed in all the groups except in the BB and CO groups. Further, a significant reduction in lymphocytes were also observed in all the experimental groups except BB (p<0.05).

Inter-group comparison at 8-month post baseline revealed that both total WBC and neutrophil counts were significantly high in DD compared to the remaining groups (p<0.05). Further, BB reported a significantly high lymphocyte counts at the same assessment point (Table. 1).

Comparison of total WBC counts at 14-month post baseline with baseline values revealed a significant reduction in the counts in groups NTM, BB, HW and CO. However, the reductions in groups DD and NTMB were comparatively less when compared to the remaining groups.

At the 14-month post baseline, all the experimental groups except HW and NTM had significantly high neutrophil counts when compared to the baseline (p<0.05). When compared with the other groups, HW and NTM had significantly lower neutrophil counts at 14-month post baseline (p<0.05). A significant reduction
in lymphocytes was observed in NTMB, BB, DD, and CO groups at 14- month post baseline when compared to the baseline (p<0.05). Inter-group comparison of lymphocyte counts at 14-month post baseline showed that both NTM and HW groups had a significant elevation when compared to NTMB, DD and CO groups (p<0.05).

**Effect of drinking water on the changes in CD$^{4+}$ and CD$^{8+}$ lymphocyte subsets**

Changes in CD$^{4+}$, CD$^{8+}$ and serum cytokines; TNFα, IL-1β, and IL-6 levels in different study groups at baseline, 8- and 14- months post baseline are shown in Table 2.

At baseline, the lowest CD$^{4+}$ lymphocyte count was observed in the CO control group compared to the other groups (p < 0.05). At 14-month post baseline, a significantly high CD$^{4+}$ lymphocyte count was observed in the NTM group compared to NTMB (p < 0.05), BB, HW, and CO (p < 0.01). Group DD had the second highest CD$^{4+}$ lymphocyte count at 14- month post baseline and it was significantly higher than BB, HW and CO groups (p<0.05). No significant difference in CD$^{4+}$ lymphocyte counts were observed between NTM and DD at 14- month post baseline (p>0.05).

Table 2. Changes in CD$^{4+}$, CD$^{8+}$ at the baseline, and 14-month post baseline, serum cytokines; TNFα, IL6 and IL-1β at the baseline and 8-month post baseline in different experimental groups (Mean±SEM).
<table>
<thead>
<tr>
<th>Study Group</th>
<th>Assessment Parameters</th>
<th>Time of Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD&lt;sup&gt;4+&lt;/sup&gt;</td>
<td>CD&lt;sup&gt;8+&lt;/sup&gt;</td>
</tr>
<tr>
<td>NTM</td>
<td>3496±290</td>
<td>1270±112</td>
</tr>
<tr>
<td></td>
<td>267.7±66.2</td>
<td>42.5±29.2*</td>
</tr>
<tr>
<td>NTMB</td>
<td>3788±90.2</td>
<td>2491±196</td>
</tr>
<tr>
<td></td>
<td>3059±319</td>
<td>1082±264</td>
</tr>
<tr>
<td>DD</td>
<td>3420±108</td>
<td>1353±166</td>
</tr>
<tr>
<td></td>
<td>2671±233</td>
<td>874±121</td>
</tr>
<tr>
<td>HW</td>
<td>2748±287</td>
<td>1491±161</td>
</tr>
<tr>
<td>CO</td>
<td>2177±212</td>
<td>1155±212</td>
</tr>
</tbody>
</table>

At baseline, rats that ingesting water from NTM had significantly higher CD<sup>8+</sup> lymphocyte counts compared to the other groups (p < 0.001). No significant difference was observed between other groups at baseline. At 14-month post baseline, a significantly high CD<sup>8+</sup> lymphocyte count was observed in the NTM group compared to NTMB, DD, BB, HW and CO groups (p < 0.01). Rats ingesting water from BB had significantly low CD<sup>8+</sup> lymphocyte count when compared to rats who ingested water from NTM and HW.

**Effect of drinking water on the changes in serum cytokines; TNFα, IL-1β, and IL-6.**

Serum TNFα, IL-1β and IL-6 levels in all test groups along with controls were assessed at baseline and 8-months post baseline and the comparison was carried out between the two assessment points.
At baseline, both serum IL-1β and IL-6 were measurable in serum, whereas serum TNFα levels remained undetected in all groups. The cytokines fluctuated differently between the two assessment points and the difference was evident among some of the test groups at 8-month post baseline (Table 2).

There was an overall reduction in both serum IL-1β and IL-6 between the two assessment points in all the test groups. A significant reduction in IL-1β was observed in both DD and BB groups while IL-6 reduction was significant in both NTM and NTMB groups when compared to the remaining experimental groups.

Serum IL-1β levels 8-month post baseline showed significantly higher levels in the NTMB and CO groups in comparison to the remaining groups, thus indicating a lower reduction between the two assessment points (Table 2).

Serum TNFα levels in all the experimental groups were not detected at baseline. However, there was overall increase in the levels at 8-month post baseline, with both DD and BB groups showing a significant surge when compared to the remaining groups (p < 0.05).

Effect of drinking water on the changes in liver cytokines; TNFα, IL-1β, and IL-6

TNFα, IL-1β and IL-6 in all experimental groups were assessed at 14-month post baseline and are shown in Table 3. Both TNFα and IL-6 levels in the DD group were significantly elevated when compared to the remaining groups. No significant difference was observed in IL-1β levels among these groups.

Effect of drinking water on the changes in serum enzymes; AST and ALT

Both AST and ALT levels at baseline were consistent in all the experimental groups with an average value of 87.33U/L ±20.06, and 33.51U/L ±2.83, respectively. There was an early increase in both AST and ALT levels among rats in NTM group and such elevation was evident up to 8-month post baseline. However, this elevation in both AST and ALT levels was not maintained at the 14-month post baseline. In NTMB group, a similar pattern of AST and ALT fluctuation was also observed (Table 3).

Table 3 Changes in serum AST, ALT at the baseline, 8- and 14-month post baseline and hepatic cytokines; TNFα, IL6, IL-1β at the 14-month post baseline in different experimental groups (Mean±SEM).
<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Assessment Parameter</th>
<th>Time of the assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT (U/L)</td>
<td>AST (U/L)</td>
</tr>
<tr>
<td>NTM</td>
<td>33.51 ± 2.83</td>
<td>87.33 ± 20.1</td>
</tr>
<tr>
<td></td>
<td>40.85±1</td>
<td>115.93±8</td>
</tr>
<tr>
<td></td>
<td>38.70±4.5</td>
<td>97.75±8</td>
</tr>
<tr>
<td>NTMB</td>
<td>44.86±2</td>
<td>214.41±9*</td>
</tr>
<tr>
<td></td>
<td>43.25±5</td>
<td>92.30±6</td>
</tr>
<tr>
<td>DD</td>
<td>43.60±1</td>
<td>152.80±7*</td>
</tr>
<tr>
<td></td>
<td>30.18±2</td>
<td>93.58±16</td>
</tr>
<tr>
<td>BB</td>
<td>43.78±3</td>
<td>87.09±4</td>
</tr>
<tr>
<td></td>
<td>36.73±1</td>
<td>92.40±2</td>
</tr>
<tr>
<td>HW</td>
<td>49.51±4</td>
<td>203.83±1*</td>
</tr>
<tr>
<td></td>
<td>123.5±3*</td>
<td>175.80±2*</td>
</tr>
<tr>
<td>CO</td>
<td>42.32±1</td>
<td>114.23±3</td>
</tr>
<tr>
<td></td>
<td>33.68±2</td>
<td>88.41</td>
</tr>
</tbody>
</table>

Rats in DD group showed a significantly elevated AST levels at 8-month post baseline, but it was not maintained at 14-month.

Rats in the HW group showed a consistently elevated AST levels from 8-month post baseline until termination of the study at 14 months. Interestingly, ALT in the same group was also elevated at 14-month.

The remaining groups did not show any significant changes in both AST and ALT levels from baseline.

**Effect of drinking water on the changes of liver and renal histopathology**
The main histopathological change observed in renal tissues were peritubular non suppurative interstitial nephritis whilst in liver tissues, it was observed steatosis, portal tract and parenchymal inflammatory cell infiltrates as prominent lesions [11]. Non-affected to affected kidney tissue ratios are summarized in the Table 4 and the percentage severity of liver lesions are summarized in Table 5.

Table 4 Mean non-affected to affected renal microscopic field ratios of rats that ingested water from different sources.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NTM</th>
<th>NTMB</th>
<th>BB</th>
<th>DD</th>
<th>HW</th>
<th>CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean non affected/affected microscopic field ratios of the kidney tissues</td>
<td>4.55</td>
<td>3.233</td>
<td>2.98</td>
<td>3.322</td>
<td>4.85</td>
<td>4.546</td>
</tr>
</tbody>
</table>

Table 5 Percentages of different hepatic histopathological changes observed in rat that ingested water from different sources.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Steatosis (%)</th>
<th>Inflammatory Lesions in the portal tract (%)</th>
<th>Inflammatory Lesions in the parenchyma (%)</th>
<th>Inflammatory Lesions in the parenchyma &amp; portal tracts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTM</td>
<td>40%</td>
<td>80%</td>
<td>60%</td>
<td>60%</td>
</tr>
<tr>
<td>NTMB</td>
<td>33%</td>
<td>67%</td>
<td>67%</td>
<td>33%</td>
</tr>
<tr>
<td>DD</td>
<td>30%</td>
<td>90%</td>
<td>70%</td>
<td>67%</td>
</tr>
<tr>
<td>BB</td>
<td>33%</td>
<td>78%</td>
<td>56%</td>
<td>44%</td>
</tr>
<tr>
<td>HW</td>
<td>60%</td>
<td>60%</td>
<td>30%</td>
<td>20%</td>
</tr>
<tr>
<td>CO</td>
<td>50%</td>
<td>25%</td>
<td>37.5%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Discussion

As described in the introduction, rats that ingested water from high disease prevalent areas such as NTM, NTMB, BB developed chronic, mild to severe interstitial inflammatory cell infiltrates around the kidney tissues and the severity of the lesions were vary with the origin of the source of water. DD from high disease prevalent NCP reported most severe tissue destruction and fibrosis in the kidney tissues compared to other groups. Rats that ingested water from low disease prevalent HW from NCP also had mild peritubular chronic interstitial inflammatory foci whilst rats that ingested water from low disease prevalent CO also reported a very mild inflammatory focus around their kidney tubules [11].

When analyzed their haematological profiles, fluctuations of WBC, DC, CD$^{4+}$ and CD$^{8+}$ counts were observed in most of the experimental groups compared to their baseline values and this can be associated with the
age-related variations observed in Wistar rats as proven by many animal studies [16, 17] & (Table 1).

Rats that ingested water from NTM showed high CD$^{4+}$ and CD$^{8+}$ counts in their blood after 14 months ingestion of water and this is well corelated with the severe kidney lesions observed in their kidney tissues. Rats that ingested water from NTMB had significantly low CD$^{4+}$ and CD$^{8+}$ cell counts in their blood and significantly low microalbumen: creatinine ratios in their urine compared to NTM [11]. This clinical picture was well corelated with the comparatively low tubular lesion index observed in rats that ingested water from NTM (Table 4). The second highest CD$^{4+}$ cell counts were observed in rats that ingested water from DD compared to other groups whilst no significant difference was observed compared to NTM. However, rats that ingested water from DD reported significantly low CD$^{8+}$ cell counts compared to NTM expressing different clinical picture at the haematology. Severely affected kidney lesions were observed in rats that ingested water from DD and those lesions were different from the lesions observed in rest of the rat groups ingested water from high disease prevalent NTM, NTMB and BB. BB and the control group CO, reported significantly low CD$^{4+}$ and CD$^{8+}$ values compared to other groups. In general, rat groups which developed very severe kidney lesions had significantly high CD$^{4+}$ & CD$^{8+}$ cell counts.

Further, significantly high TNF$\alpha$ concentrations were observed in high disease prevalent areas, in comparison to the low disease prevalent HW from the same geographical location and CO from a different geographical location (Table 2). Groups CO and HW had similar immunological findings. Though there were differences in the CD$^{4+}$, CD$^{8+}$ cell counts and microalbumen: creatinine ratios between NTM and NTMB both groups had almost similar TNF$\alpha$ concentration in their serum levels after 8 months ingestion of water (Table 2). However, statistically significant differences were not observed in TNF$\alpha$ levels in tissues levels between NTM and NTMB after 14 months of experimental period. Both groups had peritubular non suppurative interstitial nephritis. However, slight differences were observed in the distribution pattern and severity of the lesions [11]. Infiltration of T cells can cause cell injury directly by cytotoxic activity or by secreting cytokines and indirectly by activating microphages. This destruction finally leads to fibrotic replacement of the damaged tissues [18].

Kidney damage observed in NTMB may be explained by the high sodium contents observed in water sources of NTM as well as NTMB, compared to other groups [11]. The sodium contents were 55 mg/dL and 57 mg/dL respectively in these two water sources. In rats that ingested water from CO, the sodium content was 2.6 mg/dL [11]. Other water bodies such as BB and HW, relatively low sodium contents were observed than NTM (26 mg/dL) in both water sources [11]. Accordingly, gradual decline of the severity of the kidney lesion index also was observed. Recent study has shown that pro-inflammatory remodeling of renal heparan sulfate (HS) can be potentiated in normotensive Wistar rats fed on a high sodium diet [19]. The HS in a proinflammatory state can bind more sodium molecules and facilitate inflammation, fibrosis and lymphangiogenesis. Rats who were fed with a high sodium diet for 2–4 weeks showed significant changes in tubulo-interstitial T-cells, myofibroblasts etc. [19]. It is possible that the high sodium content in NTM and NTMB are the main reason for severe tubular interstitial lesion index observed in these two experimental groups. Rats that ingested water from relatively low sodium contents also developed kidney lesions. Only the severity of the lesions differed among groups and different grades of tissue destruction can be
correlated to different concentration of sodium present in water bodies from high diseases prevalent areas of the NCP.

High fluoride levels were also a common occurrence of water bodies in NCP as demonstrated in our previous in vivo experimental models [10]. Wells with high sodium contents also had high fluoride contents than those wells from low disease prevalent areas whilst overlapping common clinical features of severe kidney and liver lesions in rat groups ingested water from those wells [10 &11]

However, the highest tissue destruction was observed in rats that ingested water from DD and very prominent tissue destruction and renal fibrosis were also observed in this group. Significantly high hepatic tissue TNF alpha levels were also observed in this group. The lowest concentration of sodium was observed in DD (18 mg/dL). Accordingly, high sodium content in water cannot be the sole reason for the observed kidney destruction in CKDu in the NCP. There may be other factors in the water bodies that may contributed towards the pathogenesis of CKDu

Prominent liver damage including steatosis, portal tract inflammation, parenchymal inflammation and portal tracts inflammation together with parenchymal inflammation were observed in each group of rats (Table 5). Most rats with a very high TI lesion index in kidney tissues showed high percentage of inflammatory lesions in their portal tracts as well as in the parenchyma (Table 5). Rats with low TI lesions indices (HW and CO), in comparison to the high disease prevalent areas of the NCP, had a low percentage or zero percentage of portal tract and parenchymal lesions, respectively (Table 5). However, rats that ingested boiled water from NTM had nearly 50% lower percentage of portal tract and parenchymal lesions together with lower liver steatosis, inflammatory lesions in the portal tracts of the liver (Table 5).

The possibility of having leptospirosis and Hanta viral infections in CKDu patients in the NCP have been suggested as associated factors in the disease process [20]. It is very unlikely that rats develop active hepatitis due to leptospira or hantavirus, as they are the maintenance host of these infectious agents [21]. However, rats that ingested boiled water from NTM had a lower burden of chronic active hepatitis but severe peritubular lesions were observed in kidney tissues. Heat treatment of Leptospira spirochete at 80°C -100°C demonstrated the presence of protein fragments of Leptospira in western blot analysis [22]. But at this temperature, infectivity of the organism may be destroyed. There was no significant difference observed in haematological findings such as total WBC and neutrophil counts of rats that ingested boiled and un-boiled water from the NCP (Table 2). But rats from DD with the most severe kidney lesions reported significantly high total WBC and neutrophil counts compared to other experimental groups (Table 2). However, murine models are resistant to natural leptospiral infection. Usually, mice are considered as chronic carriers of leptospiral infection, with renal colonization of Leptospira predominantly leading to interstitial nephritis or kidney failure, but mice remain asymptomatic or can have mild clinical symptoms [21]. Wistar rats for these experiments were maintained in a microbiologically controlled environment and the colonies were negative for Leptospiral infection [23]. However, the possibility of having mild leptospiral infection introduced through un-boiled dug well water from NCP water cannot be totally overlooked in this experiment.
Active growing rats were selected for our experiment and rats were frequently handled for experimental purposes and this may have been a stressful situation for them. IL1β is a pro-inflammatory cytokine. Pro-inflammatory cytokines such as IL1β are found in higher concentrations in behaviorally active animals [24]. This may explain the high concentration of serum IL1β at baseline. However, behaviorally active animals decrease their serum pro-inflammatory cytokines without accumulating it in their serum like behaviorally passive animals [24]. Subsequently, after 8 months, their serum cytokine levels were re-evaluated. During this period, they were exposed to minimum stressful conditions. Significantly increased levels of IL1β concentration were observed in rats that ingested water from NTMB compared to other groups (NTM, DD, BB, HW). However, though it was significantly high compared to other groups, the IL1β concentration after 8 months was not significantly different from the baseline value. Rats that ingested water from CO also reported equivalent concentrations of IL1β to their baseline values and the difference observed between CO and NTMB was not statistically significant. The pro-inflammatory cytokine IL1β has been identified as a key mediator of sterile inflammatory response formed by the pattern recognition receptors mediated via recruitment and activation of different domains such as Endogenous Danger Associated Molecular (EDAM) patterns released from necrotic cells activated caspase 1 through NLPR3 inflammasome and other diverse stimuli which has the potential to stimulate the inflammasome. Inflammasomes are considered as large molecular scaffold which has the capacity to recognize the cytosolic pattern receptors, adaptor proteins and caspase 1. The secretion of 1L1β from primed macrophages are totally dependent on this mechanism [25]. There may be different stress factors such as unidentified toxins present in different water sources that can trigger these mechanisms and activate IL1β secretion. Rats that ingested water from NTMB and CO developed very severe liver steatosis than other groups. NTMB water is boiled and tap water is chlorinated. As such, the reason for the liver damage cannot be explained by infectious agents. Very mild kidney lesions were observed in rats that ingested water from CO and this water is not totally devoid of toxic agents (Table 4) This may be the reason for observing high level of IL1β in NTMB and CO. However, no significant difference was observed in liver IL1β concentrations between different groups after 14 months experimental period (Table: 2).

There was a strong correlation between the TI lesion index in the kidney tissues and liver inflammation. The highest TI lesion index was observed in rats that ingested water from DD and highest percentage of portal tract (90%), parenchymal (70%) and parenchymal along with portal tract inflammation (67%) were observed in this group. The lowest TI lesion index was observed in rats that ingested water from Colombo and low portal tract (25%), parenchymal (37.5%) and parenchymal along with portal tract inflammation (0%) were observed. The NTM group also reported significantly high TI lesion index together with portal tract (80%), parenchymal (60%) and parenchymal along with portal tract inflammation (60%). Though significant difference was not observed in TI lesion index in kidney tissues between NTM and NTMB, percentage of liver lesions had a significant reduction as liver steatosis by (7%), portal tract inflammation by (13%) parenchymal inflammation by (7%) and parenchymal along with portal tract inflammation by (27%). HW showed portal tract inflammation (60%), parenchymal inflammation (30%) and parenchymal along with portal tract inflammation (20%) (Figs. 1&2). However, rats that ingested water from CO and HW reported significantly high percentage of liver steatosis compared to other groups.
Significant rise of serum TNFα levels were observed in BB and DD after 8 months of experimental period whilst TNFα levels were undetectable at the base line levels. In our study, we observed significant elevation of serum TNFα compared to their base line levels in most of the experimental groups after the 8 months experimental period. Cytokines such as TNF α has the capacity to damage kidney tissues and leads to early fibrosis in the kidney tissues (Figures. 3, 4 and 5). However, in the latter part of the experiment, elevated hepatic levels of TNFα and CD4+ and CD8+ T cells were observed. All these mediators are potential sources that have the capacity to initiate renal destruction. According to these findings, these inflammatory mediators can directly damage kidney tissues leading to fibrosis.

**Conclusion**

Kidney damage is mediated via cytokines, cytotoxic T cells, heparan sulfate triggered by various toxic elements present in the water bodies in high and low disease prevalent CKDu areas of NCP. High sodium levels observed in water bodies of NCP remained as one of the reasons to induce direct kidney damage in rats that ingested water from in high disease prevalent areas from NCP. Peritubular non suppurative interstitial cell infiltrations in kidney tissues may be secondary to early fibrosis as evident from the current study. There was a strong correlation between the burden of liver lesions and the expression of kidney tubular lesion index. Boiling of water had been able to reduce the liver burden to a certain extend. High sodium contents were observed in NTM and NTMB and this may be the reason for severe peritubular lesion index observed in these two groups. There may be other mediators which have the potential to contribute to the pathogenesis of peritubular lesions in kidney tissue in NCP water. Further, same toxic element can damage the kidney tissues by triggering different pro inflammatory cytokines and CD4+ and CD8+ cytotoxic T cells as described earlier. Assessment of CD8+ and CD4+ cells together with TNFα can be useful in early diagnosis of CKDu in NCP and subsequently, for early therapeutic interventions. However further studies are strongly recommended to confirm the findings of the current study.

**Abbreviations**

**ALT:** Alanine Amino Transferase  
**AST:** Aspartate Amino Transferase  
**APHA:** American Public Health Association  
**ANOVA:** Analysis of Variance  
**AKI:** Acute Kidney Injury  
**BUN:** Blood Uria Nitrogen  
**BB:** Bisobandaragama  
**CO:** Colombo
Ethics approval for this study was obtained from the Ethics Review Committee, MRI, Colombo 8, Sri Lanka and ethics approval number is 11/2012. All the animal experiments were conducted according to the guidelines provided by the International Council for Laboratory Animal Science (ICLAS) and experiments...
were conducted at the local settings. All the Wistar rats used in this study were originated from the breeding colonies maintained at the Medical Research Institute, Colombo Sri Lanka for research purposes.

**Availability of data and materials**

Data generated in this study are included in the main body of the manuscript

[Table 1, Table 2, Table 3 Table 4 and Table 5].

**Competing interests**

All the authors have declared that no competing interest.

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**Authours’ Contributions**

MGT prepared the study design, acquisition of data, statistical analysis, interpretation of results and writing the manuscript, R de S by acquisition of data, interpretation of results, critical review of the manuscript and editing, BPG for acquisition of data and interpretation of results, critical reviewing of the manuscript and editing CR for acquisition of data and interpretation of results, critical reviewing the paper KR by acquisition of data and WGSSK acquisition of data MMG for original research idea for animal experimentations in CKDu, study design NE for acquisition of data MIT for editing of the manuscript. All the authors have read and approved the content in the final version available in this manuscript declared no conflicts of interests.

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Consent for publication

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