A preliminary analysis of renal infiltrating cells in acute tubular injury induced by water treated with high fluoride and high hardness in rat model

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Abstract
The impact of water containing high fluoride and high hardness on kidneys of rats was investigated. Control and test water sample with high fluoride and hardness were collected from Kandy and Mihinthale regions respectively. Twelve rats were divided into 2 control and 2 test groups. One set of control and test group received normal and test water for 60 days and the other set of control and test group received same water treatment for 90 days. Rats treated with test water for 60 days were observed loss of brush border in less than 25% of tubular cells and in 90 days associated with loss of brush border in more than 25% of tubule cells resulting in acute tubular injury compared to control group. Immunohistochemical staining revealed the strong infiltration of CD3 T-cells and CD68 macrophages in test group for 90 days.

Further, Ki-67 proliferation index was also found to be significantly higher in the group treated with test water for 90 days. Consumption of water with high fluoride and high hardness for longer period may be linked to acute tubular injury with respect to disrupt kidney tubular cells, an increased proliferative activity along with increased infiltrating CD3-, CD68- and CD68-positive immunocompetent cells.

Keywords: CD3, CD 68, CKDu, High fluoride, High hardness, Ki-67.

Introduction

The Chronic Kidney Disease of Unknown Etiology (CKDu) was discovered by the Ministry of Health in the 1990s among the paddy farmers in the North Central Province (NCP) of Sri Lanka. It is a new form of chronic kidney disease (CKD) which is characterized by the absence of well recognized causes for CKD including diabetes and hypertension[1]. The incidence of CKDu has been gradually increased in last decades and is associated with high morbidity and mortality.

Many more risk factors for CKDu have been identified and most of them were environmental risk factors linked with water, food and agrochemicals[2-6]. However, they account for a smaller proportion of the disease. Among them, high fluoride levels associated with high water hardness are important contributor to the total burden of the disease. CKDu endemic areas characterize both high fluoride and high hardness areas in Sri Lanka. This was further confirmed by the hydrogeochemical investigations carried out in CKDu hotspots in Sri Lanka[7] but its relationship to CKDu is more complex.

There are many evidences which support the role for renal histopathology of CKDu, but the role of immune cells in the disease is largely unknown. The respective involvement of innate (monocytes/macrophages) and adaptive (T lymphocytes) immunity appears to be vital in the onset and persistence of renal tubular damage, inflammation and fibrosis[8]. Although these immune infiltrating cells are uncommon in healthy kidneys, their numbers increase considerably in disease[9]. Therefore, it is desirable to understand their key participant in order to identify its relationship with CKDu. Therefore, in the present study, we performed a comparative study in rats to investigate the effect of both high fluoride and high hardness in drinking water on kidney biochemistry, histopathology and renal inflammation as assessed by immunohistochemistry in a rat model.

Material and Methods

Water samples: A water sample with high fluoride (1.66ppm) associated with high hardness (364mg/L $CaCO_3$) was collected from a selected dug well in Mihinthale area, a region located in North Central Province of Sri Lanka (8 20' 57"N 80 30' 03"E) as a test water sample. Except fluoride and hardness, all the other parameters were within the allowed values. Normal water sample was collected from Kandy, a region located in Central province of Sri Lanka (7.2582° N, 80.5988° E) as the control water sample. All the parameters including fluoride (0.2ppm) and hardness (84mg/L $CaCO_3$) were under the permissible limits of WHO standards. These water samples were selected based on the regional water quality information gathered from the Department of Geology, University of Peradeniya, Sri Lanka. Prior to sampling, normal and test water samples were subjected to water quality parameters and compared with WHO standards to confirm the experimental conditions.

Animals: Experiment was carried out with healthy 12 male Wistar rats (origin; Clea Japan, Inc.) purchased from

Medical Research Institute, Sri Lanka, weighing 200±10 g and aged 4-5 weeks. They were reared under sanitary conditions and housed in polycarbonate cages at 25°C and 50% humidity on a 12-hour light/dark cycle. The rats were acclimated for one week with food and water *ad libitum* before starting the experiment. The experimental procedures were reviewed and approved by the Ethics Committee of Postgraduate Institute of Science, University of Peradeniya, Sri Lanka and the animal treatment and handling were carried out according to the International Guiding Principles for Biomedical Research Involving Animals (Council for the International Organizations of Medical Sciences 2012)[10].

Each rat was given a unique number and their initial body weights were recorded. After one week of adaptation, animals were randomly divided into 4 groups (Figure 1), 2 control and 2 test groups, each with 3 rats and water samples were introduced as daily water supply: One control group received normal water for 60 days (C1) and the other control group received normal water up to 90 days (C2). Similarly, one test group received test water sample for 60 days (T1) and the other test group received for 90 days of test water (T2).

At the end of each treatment period, body weight of each rat was recorded as final body weight and anaesthetized by CO_2 inhalation. Blood samples were collected from each animal and transferred to polyethylene tubes. The organs, liver and kidney were carefully removed and their fresh weights were recorded.

Determination of relative organ weights: The comparison of the organ weights of treated animals with untreated animals is often complicated by differences in body weights between groups. Therefore, the ratios of the organ weight to body weight were calculated to account for differences in body weights as follows:

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

Biochemical assays: Serum samples were subjected to serum creatinine (Agappe, Kerala, India) and urea (Agappe, Kerala, India) tests. The tests were performed using diagnostic kits and absorbances were measured using a spectrophotometer (Shimadzu, Kyoto, Japan).

Histological examination: Freshly dissected kidney samples were preserved in a fixation medium of 10% formalin for 24-48 hours, dehydrated in a graded alcohol series, cleared in xylene and specimens were mounted in paraffin blocks. Then tissues were sectioned into 5μm thickness slices using a microtome (Leica, Germany), stained with hematoxylin and eosin (HE) and processed for light microscopic examination[11]. The histopathology study was conducted at the Histopathology Laboratory, Department of Pathology, Faculty of Medicine, University of Peradeniya, Sri Lanka.

Immunohistochemistry: Kidneys were fixed in formalin, embedded in paraffin and cut into sections of 2 µm. After deparaffinization, endogenous peroxidase was blocked with 3% H_2O_2 for 10 minutes. Antigen retrieval was done using target retrieval solution (Dako Deutschland GmbH, Hamburg, Germany) and cooking in a pressure cooker for 2.5 min. After blocking for 10 min with 1% BSA in 50 mM, tris pH 7.4 sections were incubated overnight at 4°C using the following antibodies diluted in 1% BSA in 50 mM tris pH 7.4: CD68, a mouse monoclonal antibody against rat CD68 (clone ED-1, Bio-Rad GmbH) 1:50; CD3, a monoclonal rabbit antibody against human CD3 (Zytomed Systems GmbH, Berlin, Germany) and 1:50; Ki67, a mouse monoclonal antibody against the proliferation marker Ki67 (Dako Deutschland GmbH, Hamburg).

After washing with 50 mM tris buffer at pH 7.4 supplemented with 0.05 tween, 20 sections were incubated with 1:500 dilutions of the following secondary antibodies for 30 minutes at room temperature, a biotinylated horse anti- mouse IgG (H+L) and a biotinylated goat anti- rabbit IgG (H+L) (both purchased from Vector laboratories, Burlingame, CA, USA).

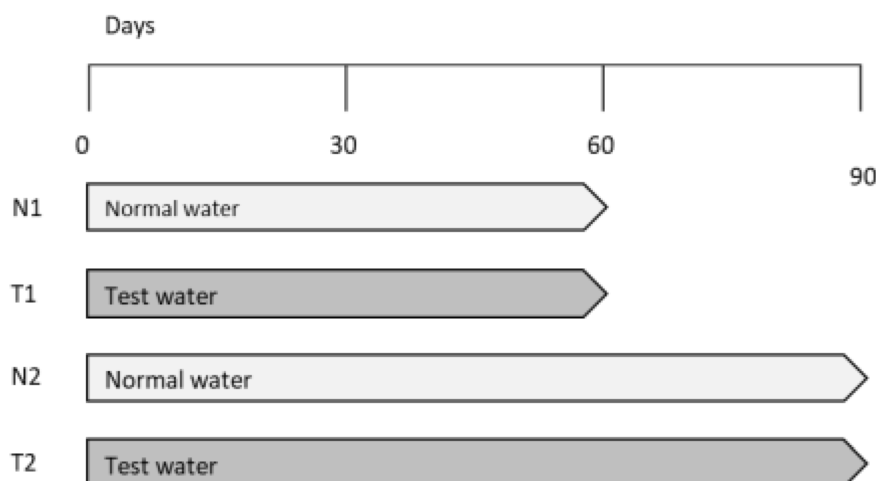

Figure 1: Schematic representation of the experimental design.

Following removal of non-bound secondary antibodies by washing with tris buffer, ABC-kit and DABImmpact as substrate (all from Vector laboratories) were applied. Negative controls for immunostaining included either deletion or substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or pre-immune rabbit IgG.

Quantitative evaluation of CD3, CD68 and Ki67-positive cells: After immunohistochemistry, CD3-positive T-cells, CD68-positive macrophages and Ki67-positive cells were analyzed by light microscopy at 40x magnification in the renal cortex. For quantitative evaluation, positive cells were counted in 12 high power fields and mean values of positive counts per high power were shown.

Statistical analysis: All values were expressed as the mean SD. Data were compared by using One-way Analysis of Variance (ANOVA). Values of $P<0.05$ were accepted as significant.

Results

No significant differences in the relative kidney weights in test water treated rats as compared to control: Kidneys were analyzed using organ-body weight ratio to evaluate the effects of normal water and test water samples on kidney weights (Figure 2). Relative kidney weights were considerably increased in test water treated groups for 60 and 90 days compared to respective control groups. But these variations were statistically not significant.

Test water administration increase serum creatinine and urea levels to a significant extent: Both serum creatinine and serum urea are widely accepted biomarkers to assess the renal functions. Therefore, we next examined whether the treatment of test water would affect these renal biomarkers. There was a slight increase in serum creatinine and urea levels in test water treated group for 60 days (Figure 3a and b). But this increase was statistically not significant. However, at 90 day, both serum creatinine and urea levels showed a significant ($p<0.05$) increase in test water treated group compared to respective control groups.

Test water induced tubular injury resulting in mild histopathological changes: Further, histological impact was observed in order to find out long term potential toxic effects in cells or tissues on precise target organs. In all treatment groups, no mortality or any adverse clinical signs of external toxicity were observed in any rats. But histopathological analysis revealed several histopathological alterations in test water treated groups compared to control groups. Kidney sections from normal water treated control rats displayed normal renal tissue structure with intact renal tubular epithelial cells and no obvious pathological changes in glomerular or renal interstitium without any degenerative changes and necrosis after 60 and 90 days (Figure 4a and b).

Morphological changes including loss of brush border in less than 25% of tubular cells, tubular epithelial degeneration and integrity of basement membrane (Figure 4c) were clearly observed in the rats treated with high fluoride associated with water hardness (T1) for 60 days. Light microscopic evaluation of kidneys in 90 days of test water treated group (T2) revealed tubular injury with loss of brush border in more than 25% of tubular cells and thickened basement membrane (Figure 4d). Despite the presence of tubular injury, the cellular debris in the proximal tubules was prominent and both shedding of few viable and necrotic cells into the tubular lumen were common.

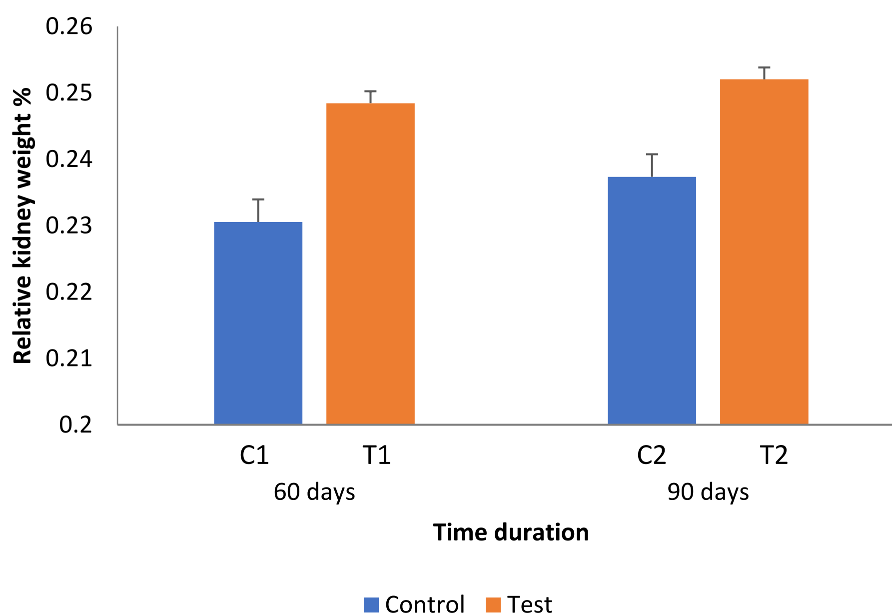

Figure 2: Relative kidney weights of rats exposure to normal water and test water for 60 and 90 days. Error bars represent standard error. Data are expressed as mean ± S.D., (n=3)

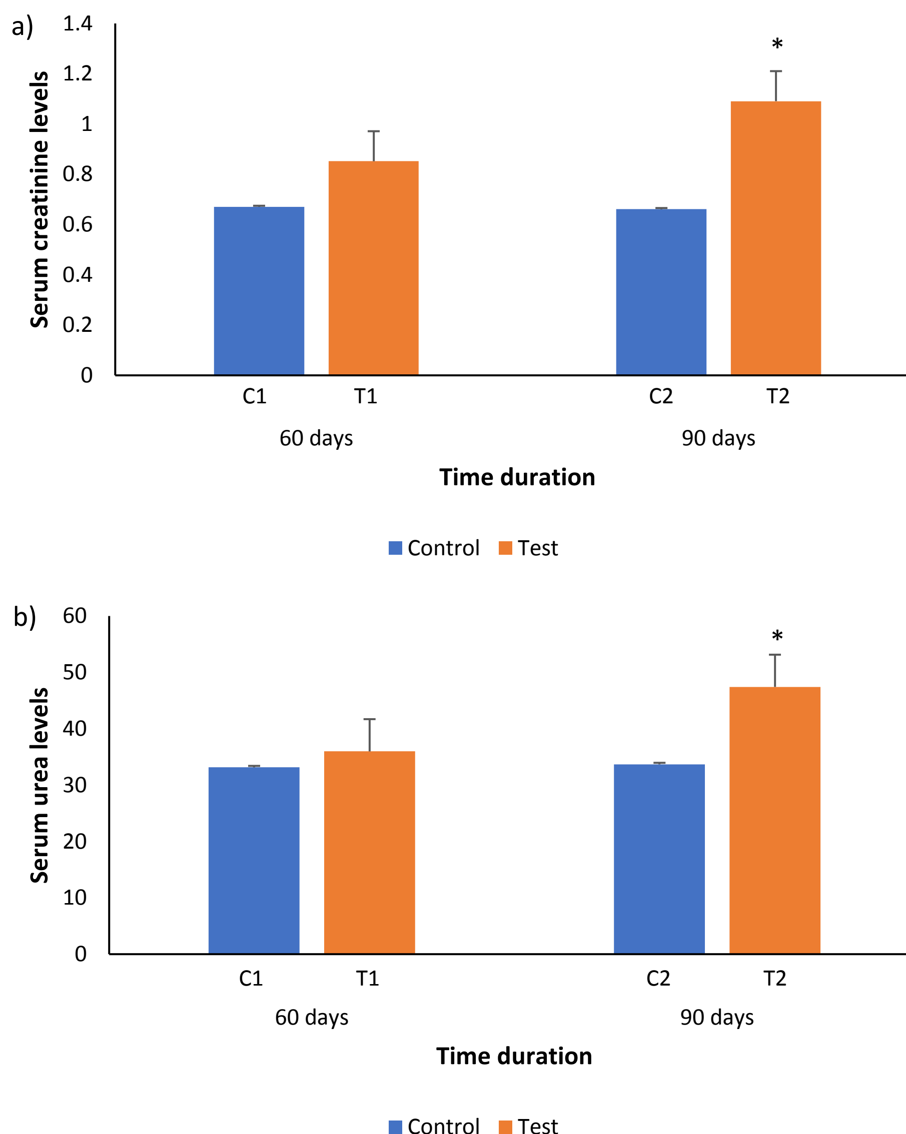

Figure 3: Effects of normal water and test water sample on a) serum creatinine levels and b) serum urea levels after 60 and 90 days of exposure. *p<0.05 compared with respective control. Error bars represent standard error. Data are expressed as mean ± S.D., (n=3)

Changes in renal inflammation and proliferation in test water treated groups on day 90: We characterized the infiltrating cell populations using CD3 (T lymphocytes) and CD68 (macrophages) immunostaining at days 60 and 90. For CD3-positive cells, we found no differences among the control and test groups treated for 60 days and both the amount are much lower than the groups in 90 days. On day 90, the amount of CD3 cells was significantly ($p<0.05$) higher in test water treated group compared to respective control group kidneys (Figure 5a).

The number of CD68 cells was higher when compared with CD3 cells. Comparing the four groups, test group treated with test water for 60 days showed significantly ($p<0.05$) less CD68 cells compared to control group (Figure 5b). However, this difference was no longer seen at day 90. On day 90, CD68 showed a significant increase ($p<0.05$) compared to respective control group.

We next examined cellular proliferation in the renal biopsies by staining for Ki-67 to quantify proliferative activity (Figure 5c). Very few proliferating intimal cells were observed in the normal water and test water treated samples for 60 days. The control group for 90 days contained some proliferating cells compared to 60 days. However, significant levels ($p<0.05$) of Ki-67-positive cells were observed in the test water treated group for 90 days compared to control group as well as 60 days.

Discussion

The organ weight is an important indicator of pathology in toxicological studies[12]. Among the organs, kidney is a major site of excretion of xenobiotics from the body which results in its tendency to exhibit chemically-induced toxicological effects at a higher rate than most other organs[13].

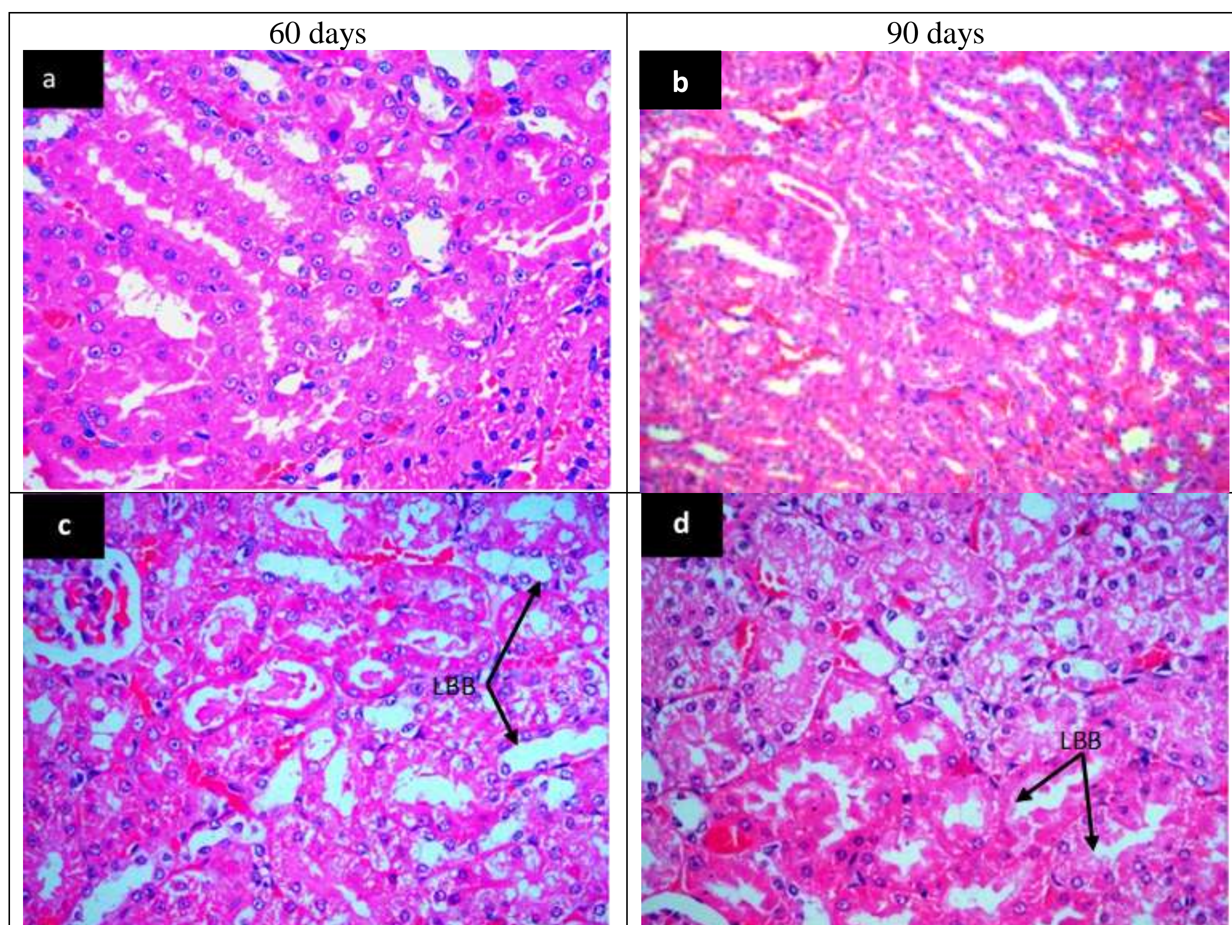

Figure 4: Histopathological examination of kidney tissues stained with hematoxylin-eosin (HE) under light microscope (200X). Control rats treated with normal water for (a) 60 days and (b) 90 days showing normal rat kidney with normal tubular brush-borders (BB); (c) tubular brush-borders loss less than 25% of tubular cells and integrity of basement membrane in test water treated rats for 60 days; (d) loss of tubular brush-border in more than 25% of tubular cells and thickened basement membrane in test water treated rats for 90 days

Our analysis showed an increase in relative kidney weights in the test groups treated with high fluoride associated with high hardness water for 60 and 90 days compared to control groups. Greaves[14] has also reported that presence of renal toxicity, tubular hypertrophy, or chronic progressive nephropathy might be reflected by the changes in kidney weight.

Although the increment is statistically not significant, relative weights were correlated with the renal biomarkers, serum creatinine and urea seen in the test groups. Among many biochemical parameters in blood, serum creatinine and urea are indicating as more sensitive markers for the detection of the renal function[15].

In our study, test water that contains high fluoride and hardness influences the serum creatine and urea levels with a significant increase at 90 days indicating a renal damage. Many studies have shown that calcium supplementation caused great increase in renal biomarkers[16,17].

The histological results of this study showed that administration of high fluoride associated with high hardness water for 60 and 90 days caused morphological injuries including loss of brush border in tubular cells, tubular epithelial degeneration and integrity of basal membrane. Loss of brush border indicates proximal tubular cell damage and the tubule cell damage and cell death characterizes acute tubular injury usually resulting from an acute ischemic or toxic event[18].

Based on some research publications, renal tubular damage initiated at an early stage of CKDu. According to Nanayakkara et al[19], renal tubular damage is visible at the very early stage in the disease pathological process and progresses as the disease advances. In our previous study, fluoride alone has not shown any renal histopathological changes even at higher doses[6]. Calcium plays a critical role in many cell functions and it has a high affinity with fluoride ions and its binding with calcium causes ectopic calcification in teeth, bone and soft tissues[20].

On the other hand, higher Ca^{2+} levels which cause low Na^+/Ca^{2+} ratios cause the damage on kidney tubular cells in the presence of fluoride by forming calcium fluoride (CaF_2) which is insoluble in water[3].

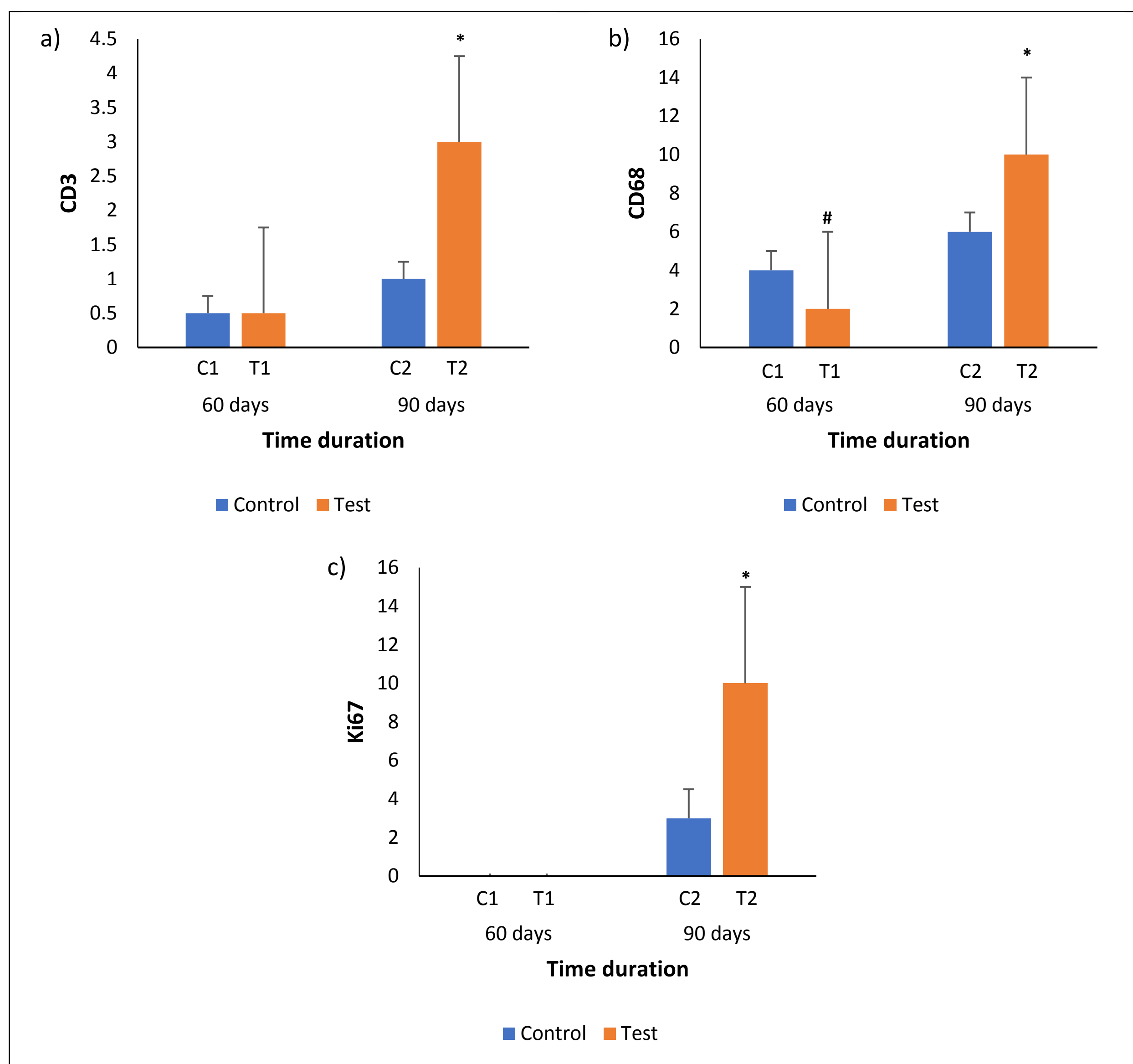

Figure 5: Cell counts of a) CD3, b) CD68 and c) Ki67 from immunofluorescence staining in normal and test water treated groups for 60 and 90 days. $^{\#}p<0.05$ compared with C1; $^{*}p<0.05$ compared with C2

Some studies have reported that fluoride increases intracellular (plasma membrane, mitochondria, endoplasmic reticulum and cytoskeleton) and causes renal calcification in rat renal epithelial cells resulting in tubular cell calcium overload which ultimately results in cell injury and death[21-23].

Macrophages are found in elevated amounts in unhealthy kidneys than in normal kidneys, where they act as key players in renal injury, inflammation and fibrosis[24]. Macrophages belong to the family of mononuclear phagocytes and are considered to originate from a common myeloid progenitor in the bone marrow.

Macrophages perform a wide range of critical roles in homeostasis, surveillance, immune response and tissue injury and repair. In a tissue injury, blood monocytes are engaged to the damaged sites and differentiate in response to microenvironment signals to which they are exposed. The degree of macrophage infiltration is correlating with the degree of renal injury[25].

Therefore, this will cover research into the role of macrophages in renal injury. The present study of immunohistochemical staining demonstrated that T lymphocytes, as assessed by CD3 and macrophage as assessed by CD68, that infiltrate the kidney, can participate in the acute tubular injury induced by high fluoride associate with high hardness resulting in increased frequency in renal biopsy tissues. CD3+ T cells were the major infiltrating cells in the kidney and CD68 is a pan-macrophage marker[26]. The role of T cells in tissue injury is supported by several early studies[27]. Discovery of cell proliferation index is encompassing a wide array of research including experimental and clinical trials[28].

According to our results, Ki-67 expressing cells were found to be significantly higher in the high fluoride and high

hardness water treated group at 90 days. The anti-Ki-67 monoclonal antibody is directed to a nuclear antigen only found in proliferative cells and is expressed during all active phases of the cell cycle (Gap 1, synthesis, Gap 2 and mitosis), but is absent in resting (G0) cells[29]. It denotes as an excellent and reliable marker for identifying cells that are in the growth cycle. Proliferating cells are present in the proximal tubules, distal tubules, glomeruli etc. and some animal studies have shown that cells in the tubules regenerate in response to renal injury[30,31].

Conclusion

Acute tubular injury induced by high fluoride associated with high water hardness is characterized by elevated serum creatinine and urea levels with increased numbers of infiltrating cells namely CD3- and CD68-positive cells and increased renal proliferation. Therefore, CKDu induced by high fluoride associated with hard water may be mediated by inflammatory cells but requires further investigations.

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