AMELIORATION BY JAMBUL FRUIT EXTRACT OF FLUORIDE-INDUCED HEPATO-NEPHRONAL HISTOPATHOLOGIES AND IMPAIRED NEUROMOTOR CAPACITY IN MICE

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ABSTRACT: We studied the amelioration potential of jambul (*Syzygium cumini*) fruit extract (JE) on fluoride ion (F) mediated behavioral alterations and hepato-nephronal histopathologies in four groups (n=10 per group) of adult male mice. Two groups, control (C) and jambul (J), were fed F-free water for 15 days while the other two groups, NaF (F) and NaF + jambul (F+J), were given 50 ppm F (from NaF) in drinking water on days 1–10 and F-free water + 0.25mL JE 12-hourly on days 11–15. The daily water intake in each group was recorded for the first 10 days. All animals were subjected to performance behaviors (balance beam bar, orientation, and climbing activities) on days 11–15 (post F-exposure period) and sacrificed to recover liver and kidneys on day 16. The organs were processed for microtomy and H & E staining. The daily water intake throughout the F-exposure period (days 1–10) in C and J groups remained constant apart from minute fluctuations while a persistent gradual decline was observed in F and F+J group animals. The mean water consumption (mL/g body weight/day) was significantly lower in F (0.0878±0.00239) and F+J (0.0873±0.00238) than in C (0.1231±000.597) and J (0.1229±0.0006) groups. Although the mean time taken to complete the balance beam bar, orientation, and climbing activities decreased gradually during the period of study (days 11–15) in all 4 groups, it remained persistently higher in F and F+J groups than in C and J groups. Hepatic histopathologies observed in F group animals included cytoplasmic vacuolations and disfigured nuclei of the hepatocytes, misaligned hepatic cords, and enlarged sinusoids and central lobular veins. Reversal of these pathological signs, with hepatic tissue regeneration and rehabilitation of the hepato-lobular arrangement, was seen in F+J group. No obvious signs of pathology were seen in histological sections of kidney in F group. Micrometric data revealed significantly higher ($p \leq 0.05$) mean percent fractional weight of liver and cross-sectional area (CSA) of hepatocytes in F (6.23±0.32 g/100 g bw and 317.26±12.08 μ^2, respectively) than C (5.3±0.25 g/100 g bw and 270.09±9.1 μ^2), J (5.15±0.19 g/100 g bw, and 247.37±7.24 μ^2) and F+J (5.72±0.15 g/100 g bw, and 179.21± 5.9μ^2) groups. The nephronal micrometric data for the endothelial brush border thickness of the proximal tubules and the CSAs of glomeruli and proximal tubules showed significantly lower mean values in F (4.92±0.38 µ, 1712.9±97.6 μ^2, and 816.8±34.88 μ^2, respectively) as compared to C (6.66±0.17 µ, 2265.8±110 μ^2, and 1283±66.12 μ^2), J (6.92±0.13 µ, 1898±113.2 μ^2, and 1097.3±35.3 μ^2) and F+J (6.91±0.16 µ, 1841.6±95.3 μ^2, and 1042.4±43.3 μ^2) groups. Conversely the mean luminal CSA of the proximal tubules was significantly increased in F (199.6±14.06 μ^2) compared to C (123.1±5.56 μ^2), J (88.12±5.25 μ^2), and F+J (119.1±6.94 μ^2) groups. The results showed that fluoride exposure in mice leads to various hepato-renal histopathologies and alterations in performance behaviors. These toxicities were convincingly ameliorated with JE treatment.

Keywords: Amelioration of F toxicity; Fluoride toxicity; Hepato-nephronal pathology; Neuro-motor capacity; *Syzygium cumini* (Jambul).

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INTRODUCTION

An imbalanced dietary minerals intake has been reported to cause histopathological changes in murine liver and kidney.[1] The fluoride ion (F) is a common water contaminant that, in addition to damaging skeletal tissue and teeth, can also impair major body organs including liver, kidney, brain, pancreas, and testis.[1-8] The biochemical aspects of F toxicity on lipid peroxidation, necrosis, and inflammation of liver and kidneys have been studied.[9] In young rats morphological changes of hepatocytes on F exposure have been reported.[10] Moreover, F exposure has been reported to cause neuronal damage in the hippocampus of the mouse brain,[11] the part of central nervous system believed to be involved in memory, learning, emotions, and autonomic activities.[12] F exposure, in a dose of 20 mg/kg body weight (bw) in mice for 14 days, resulted in increased F levels in brain and gastrocnemius muscle, the major weight bearing muscle of the (lower) leg, and caused significant alterations in the cytoplasmic antioxidant enzyme system and the membranous Na^{+}-K^{+}-, Ca^{++}-, and Mg^{++}-ATPases and acetylcholine esterase activities in these organs.[13] Jambul *(Syzygium cumini)* is a rich source of phenolic compounds with antioxidant and anti-inflammatory properties.[14] The bright purple color of jambul fruit is due to the presence of different anthocyanins (cyanidin, malvidin, petunidin, delphinidin, peonidin, and glucoside) which have potent antioxidant capacities. Also present in jambul pulp, apart from anthocyanins, are tannins, vitamin C, gallic acid, ellagic acid, ellagi-tannins, steroids, flavonoids, terpenoids, fatty acids, phenols, minerals, and different vitamins.[15-20] Because of these precious components jambul fruit extract (JE) possesses a wide range of pharmacological properties including anti-bacterial, anti-fungal, anti-viral, anti-allergic, anti-arthritic, anti-pyretic, anti-cancer, anti-diarrhoeal, anti-plaque, anti-genotoxic, anti-ulcerogenic, anti-diabetic, anti-proliferative, cardio-protective, chemo-preventive, radio-protective, hepato-protective, nephro-protective, neuro-psycho-pharmacological, free radical scavenging, anti-infertility, and hypoglycemic effects.[19-22] In continuation of our previous research,[2] in the present study we investigated the mitigating effects of JE on F histo-pathologies of liver and kidney and the neuro-muscular capacity in mice.

MATERIALS AND METHODS

Preparations and requirements and protocols: The animals (adult male mice) used, housing conditions (12–12 hr dark light cycles, 45% humidity and 23±3ºC), treatment groups, [control (C), NaF (F), jambul (J), and NaF + jambul (NaF+J)], group size (n=10), preparation of treatment materials [NaF (50 ppm) solution and JE], laboratory preparations (histological work), chemicals (NaF, ethanol, xylene, picric acid, glacial acetic acid, formaldehyde, embedding wax, Canada balsam, and hematoxylin and eosin, etc.) and the instruments (scissors, forceps, scalpels, camera fitted microscope, microtome, and digital balance, etc.), glassware (specimen vials, histological slides, and cover slips etc.), software (CorelDRAW11, and SPSS20) and protocols (animal maintenance, treatments, and

recovery) employed remained the same as described in the previous publication of this group.[2]

Behavioral studies: General performance behaviors, involving (i) the time taken to orient to an upright position on a vertically placed mesh board, (ii) the climbing activities time to reach to the top of the mesh board, and (iii) the time taken to complete balance beam bar activities, were studied only during the post F exposure period (11–15 days). All general behaviors and activities tests were observed at the beginning of the light period for each day.

Orientation and climbing activities: Each animal was carefully placed to cling on a vertically placed, wooden framed, metal mesh board, 50 cm high and 30 cm wide, in a head-down position below a horizontal line in the middle, 25 cm from the top. The times taken to orient to an upright position (head oriented vertically in an upright position) and to climb to the top of the mesh board were recorded.

Balance beam activity: Each animal was placed in the middle of a beam, 100 cm long and 1.5 cm wide, suspended horizontally 10 cm above the ground by wooden stands placed at both ends. The walking time to reach either of the two ends of the beam bar was recorded.

Organ recoveries: Liver and kidneys were obtained from each animal at the end of the study period (16^{th} day). The percent fractional weight of liver and kidney was calculated separately for each animal employing following formula.

$$\text{Percent fractional weight} = \frac{\text{Organ weight}}{\text{Body weight}} \times 100$$

Histological observations: Histological sections (5 μ thick) of both tissues (kidney and liver) were observed on a trinocular research microscope (Labomid CXR_2) and digital photographs were obtained at various magnifications (100, 400, and 1000 ×**)** using a 7.2 MP digital camera (Sony DSC-W35) mechanically attached to the microscope. The digital photographs of selected sections from all four groups were processed in CorelDRAW11 for cropping, color, contrast, and labeling, etc.

Micrometery and data analysis: For the micrometric data, digital snapshots (400 ×) were obtained from the histological sections of each animal of 10 randomly selected (i) hepatocytes, (ii) glomeruli, and (iii) cross-sections of the proximal nephronal tubules which showed the internal cavity and brush border. The diameters of above mentioned structures (except brush border) were obtained using two right angled lines passing through the center of these structures digitally using a pre-calibrated digital scale (obtained from the photographs of a stage micrometer on the fixed camera and microscope specifications) in CorelDRAW11. The cross-sectional area (CSA) was finally calculated using following formula.

$$\text{Cross-sectional area} = \frac{(\text{Length} \times \text{Width})}{4} \times \pi$$

The brush border thickness for a tubular section was measured from four random places and their mean was treated as the thickness of that tubule. Means of 10 values for each parameter were used as the unit value for each animal. The 10 unit values in each case were used to obtain group means±SEM (Table 2) and for the further statistical analysis (ANOVA and Duncan's Multiple Range Test).

RESULTS

Daily water consumption: The data obtained indicated a persistent lower water intake (mL/g bw/day) in the F and F+J groups compared to that of the J and C groups (Figure 1). Analysis of variance for the daily water consumption data among the groups for the entire period of F exposure (10 days) showed a highly significant variation among the groups ($p<0.001$). The post hoc analysis showed that the C and J groups differed significantly ($p\leq0.05$) from the F and F+J groups.

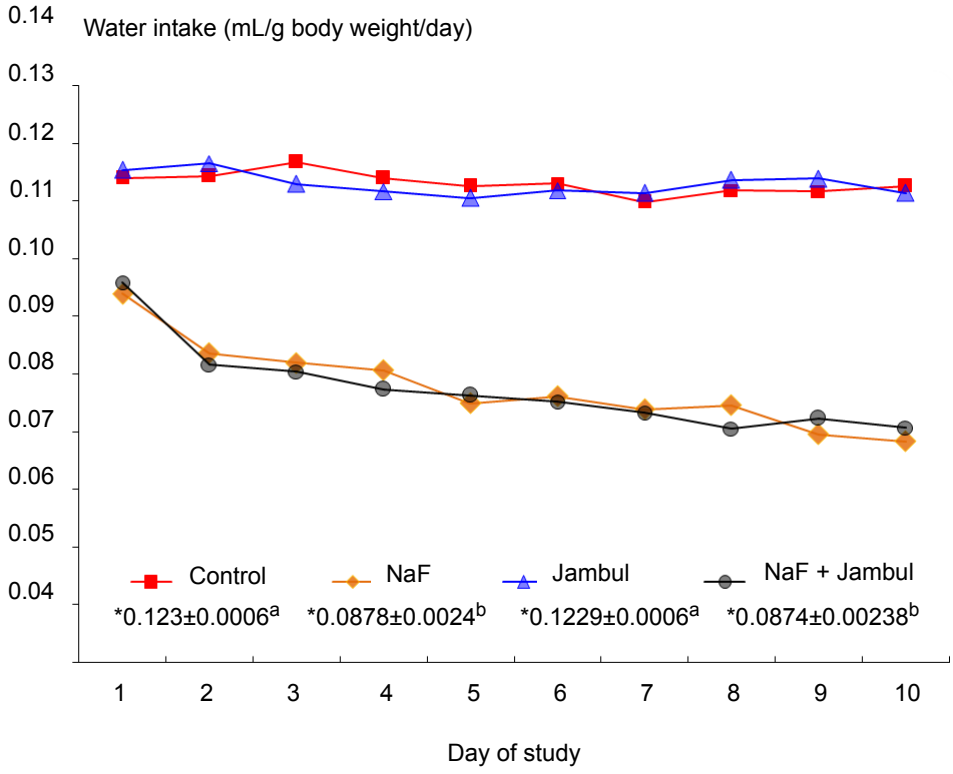

Figure 1. Line graph showing the mean daily water consumption among the groups.
*Average water consumption (mL/g body weight/day)±SEM for the entire period of F exposure (1–10 days).
[ab]Groups not sharing a lowercase letter differed significantly ($p\leq0.05$) from each other.

Balance beam bar activity: Although there was a gradual decline in the mean time taken to complete the balance beam bar activity in all the groups during the period of study (11–15 days), the average time taken to complete the activity remained highest in the F group followed by the F+J, C, and J groups in descending order (Figure 2).

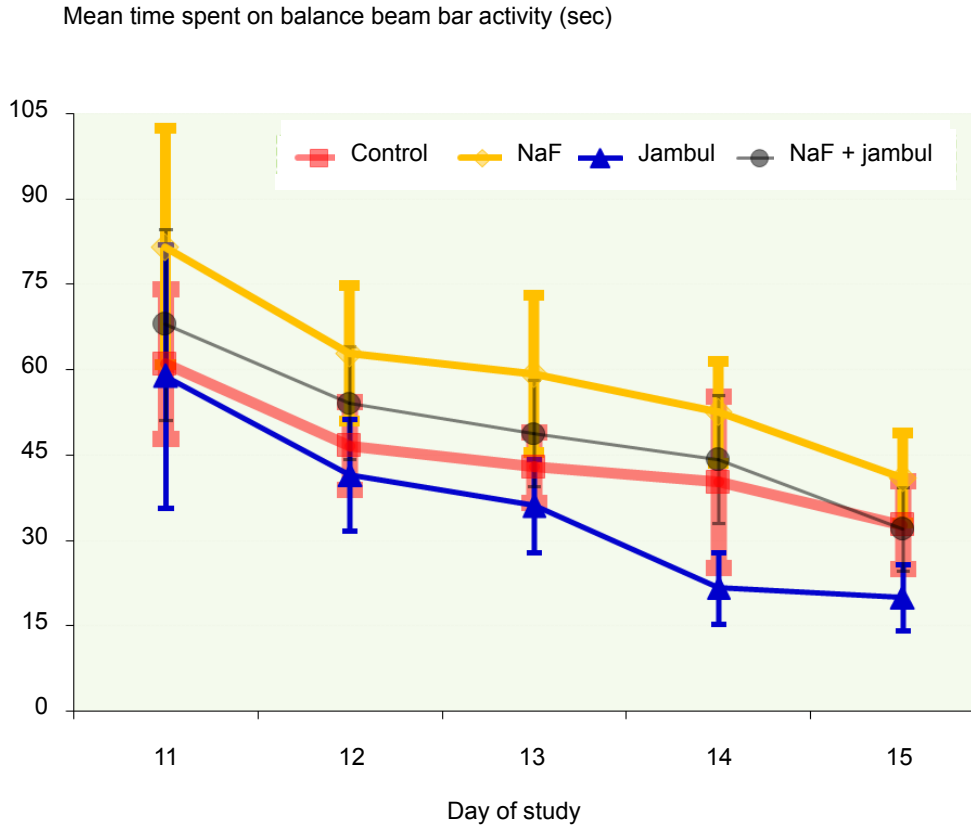

Figure 2. Line graph showing the mean time spent to complete the balance beam bar activity during the post F exposure period (11–15 days).

Orientation time: The longest mean time taken to orient to a vertically upright position was taken by the F group mice followed by the mice in the F+J, C, and J groups (Figure 3).

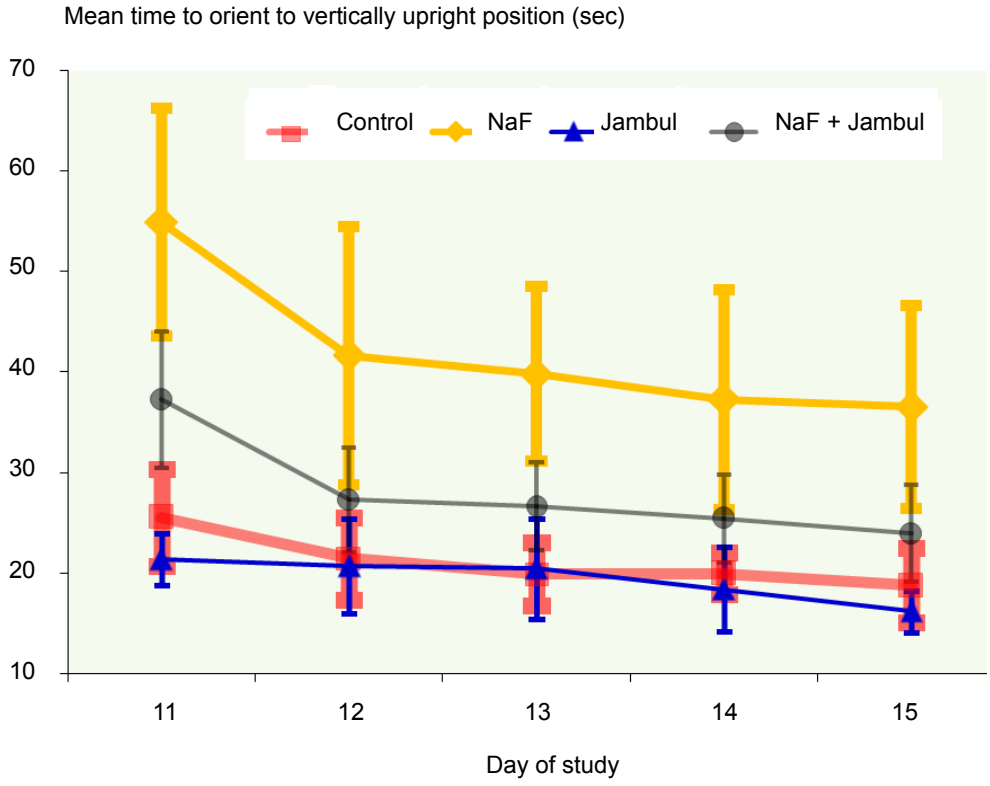

Figure 3. Line graph showing the mean time (sec) to orient to a vertically upright position to start the climbing activity during the post F exposure period (11–15 days).

Climbing time: The mean time taken to move up to the top edge of the vertical mesh board in the experimental groups reiterated the pattern for the orientation time (Figure 4).

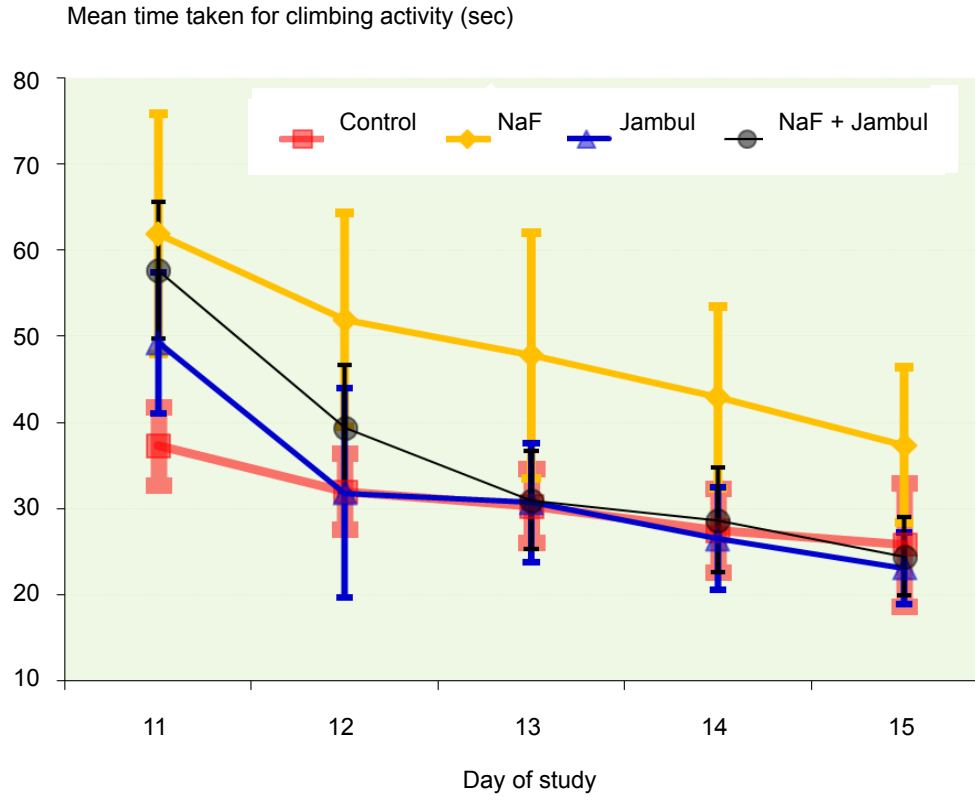

Figure 4. Line graph showing the mean time (sec) taken to move up on the top edge of the vertical mesh board during the post F exposure period (11–15 days)

Percent fractional weight of liver and kidney: Highest to lowest mean percent fractional liver weights (g/100 g bw) of liver were recorded in the F, F+J, C, and J groups. Statistical analysis showed a significant ($p<0.05$) difference among the groups. The post hoc analysis showed a significantly higher mean percent fractional weight of liver in the F group compared to the other three groups ($p\leq0.05$). Moreover, the J and F+J groups also differed significantly from each other ($p\leq0.05$). Conversely, with slight variations, the mean fractional weight of the kidneys in all the groups remained constant (Table 1).

Table 1. Mean percent fractional weights (g/100 g body weight) of liver and kidneys (Values are means±SEM)

Percent fractional weight	Groups			
	Control	NaF	Jambul	NaF+Jambul
Liver	5.3±0.25[ab]	6.23±0.32[c]	5.15±0.19[a]	5.72±0.15[b]
Kidney	1.66±0.07	1.51±0.09	1.59±0.1	1.67±0.07

[abc] Any two groups not sharing a common lowercase letter differed significantly ($p \leq 0.05$) from each other.

Liver histology and micrometry: The histological slides in the control group showed well placed single cell thick hepatic cords consisting of mono-nucleated and bi-nucleated hepatocytes (a characteristic feature of rodents) lined with Kupffer cells interposed by hepatic sinusoids (Figure 5A).

In the F group slides the presence of hepatocellular debris along the centrilobular veins and cytoplasmic vacuolations (leading to increased hepatocellular size), with, simultaneously, diffused and disfigured nuclear dimensions (indicative of necrosis) of the surviving hepatocytes, caused an irregular distribution of the hepatic cords and led to squeezed sinusoids (Figure 5C).

In contrast to the situation in the F group, the slides of the J group showed clearly visible sinusoids and no signs of peri-central debris. The hepatocytes showed denser cytoplast with centrally placed rounded nuclei (Figure 5B).

In the F+J group the liver slides showed scattered aggregations of the hepatic stem cells with oval nuclei and the epithelial cells (presumptive Kupffer cells) were clearly observed. Moreover, there were signs of recovery and regeneration of the hepatolobular architecture such as the appearance of proliferating hepatoblasts and their progressive differentiation into hepatocytes, cells having dense, rounded, compact, and darkly stained nuclei surrounded by a thin layer of cytoplasm. This group also showed signs of alignment by these regenerated hepatocytes to rehabilitate the nascent hepatic cords (Figure 5D).

Micrometric data have showed the highest mean CSA value of the hepatocytes was in the F group, followed by the C, J, and F+J groups in descending order. Statistical analysis indicated a highly significant variation ($p<0.001$) among the groups. Post-hoc analysis showed that the F and F+J groups differed significantly ($p \leq 0.05$) from each other as well as from the C and J groups (Table 2).

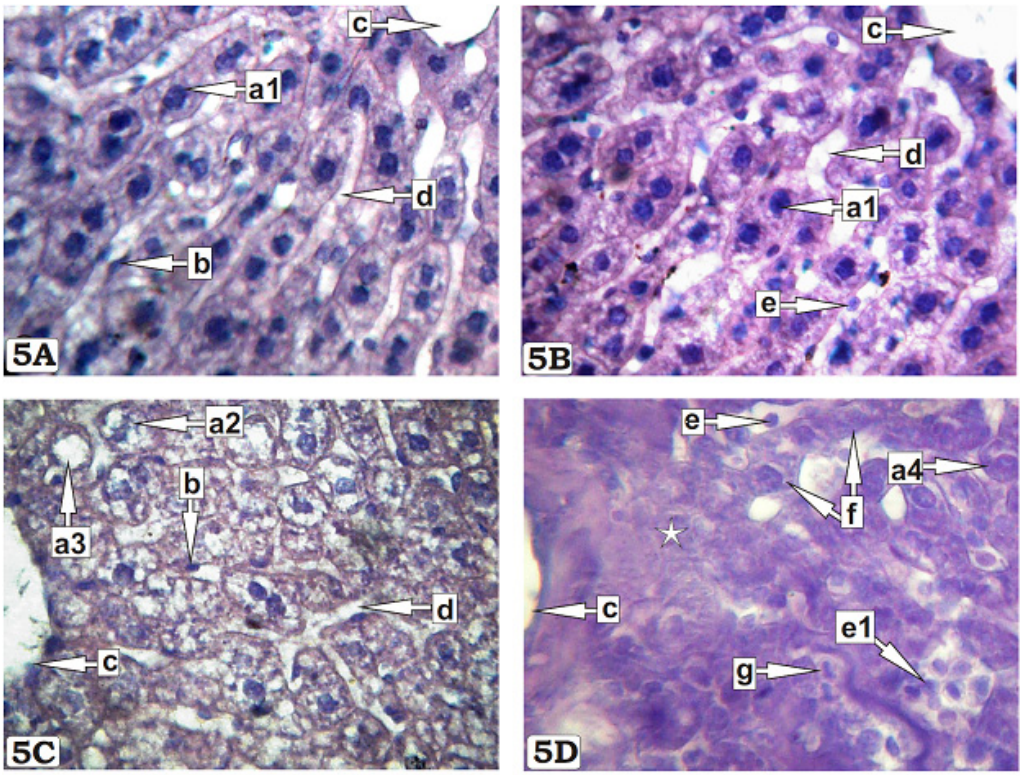

Figure 5. Histological sections of liver (400 ×). A: control, B: jambul, C: NaF, and D: NaF+ jambul treated groups. a1: Normal hepatocyte, a2: hepatocyte apoptosis (with disfigured nucleus), a3: apoptosed hepatocyte, a4: differentiating hepatocyte with dense nucleus, b: Kupffer cell, c: centri-lobular vein, d: sinusoid, e: hepatoblast, e1: aggregation of hepatoblastic stem cells, f: differentiating hepatic cord, g: epithelial cell, white star: hepatocytic debris.

Table 2. Micrometry of the hepatocytes and various parameters of the nephrons (Values are means±SEM)

Micrometric parameters	Groups			
	Control	NaF	Jambul	NaF + jambul
Cross-sectional area (CSA) hepatocyte	270.09 μ^2±9.1^{a}	317.3 μ^2±12.08^{b}	247.4 μ^2±7.24^{a}	179.2 μ^2± 5.9^{c}
CSA glomerulus	2265.8 μ^2±110^{a}	1712.9 μ^2±97.6^{b}	1898 μ^2±113.2^{c}	1841.6 μ^2±95.3^{c}
CSA proximal tubule (nephron)	1283 μ^2±66.12^{a}	816.8 μ^2±34.88^{b}	1097.3 μ^2±35.3^{c}	1042.4 μ^2±43.3^{c}
Luminal CSA of the proximal tubules	123.1 μ^2±5.56^{a}	199.6 μ^2±14.06^{b}	88.12 μ^2±5.25^{c}	119.1 μ^2±6.94^{ac}
Thickness of the proximal tubular brush border	6.66 µ±0.17^{a}	4.92 µ±0.38^{b}	6.92 µ±0.13^{a}	6.91 µ±0.16^{a}

^{abc}Any two groups not sharing a common lowercase letter differed significantly ($p \leq 0.05$) from each other.

Renal histology and micrometry: The renal histopathological effects of F treatment were more subtle in nature and no major structural alterations were observed. Micrometric data revealed the highest mean CSA of glomeruli was in the C group followed by the J, F+J, and F groups in descending order (Table 2). Analysis of the data showed an overall highly significant variation ($p<0.01$) while the post hoc analysis indicated a significant ($p\leq0.05$) decrease in the glomerular CSA in the F group compared to the C, J, and F+J groups (Table 2).

The group means of the CSA of the proximal tubules also showed the same trend (i.e., from highest to lowest C, J, F+J, and F respectively). Statistical analysis showed a highly significant ($p<0.001$) variation among the groups. The post hoc analysis indicated a significant ($p\leq0.05$) variation between the C and F groups while these two groups also differed significantly from the J and F+J groups (Table 2).

In contrast to the tubules CSA, the maximum mean luminal CSA of the proximal tubules was observed in the F group followed by the C, F+J, and J groups in descending order. Statistical analysis indicated a highly significant ($p<0.001$) variation among the groups. The post hoc analysis indicated a significant ($p\leq0.05$) difference among C, F, and J groups while the F+J group differed significantly from the F group only (Table 2).

Interestingly, the highest mean value of the thickness of the luminal ciliated brush border of the proximal nephronal tubules was observed in the J group followed by the F+J, C and F groups. Statistical analysis showed a highly significant ($p<0.001$) variation among the groups. The post-hoc analysis indicated a significant ($p\leq0.05$) variation of the F group compared with the other three groups (Table 2).

DISCUSSION

A significant decrease in water consumption in the F exposure groups (F and F+J) compared to the non-exposure groups (C and J) indicates a probable unpleasant change of taste with dissolving F in drinking water.[23] Fluoride has now almost been established as an environmental agent with potent neurotoxic outcomes.[11-13,24] Its long term exposure (100–300 ppm for 12 weeks) in rats was found to cause a marked reduction in aggression and boxing bouts.[25] This indicates that chronic or sub-chronic F exposure can cause performance lethargy that may be attributable to its neuro-motor toxicity[24] and partly to the loss of musculoskeletal strength.[26] The results obtained in the present study, indicating a much higher mean time taken to complete performance activities (balance beam bar, orientation and climbing activities), are thus consistent with this literature.

The significant increase in the percent fractional weight of liver and the mean CSA of the hepatocytes in the F group clearly indicates the occurrence of hepatic inflammation with fluoride exposure.[27] The hepatic histopathological signs of F exposure, like vacuolar formation, increased cellular size, and disfigured nuclei of the hepatocytes (indicating necrosis), sequestered sinusoids, and distortion of hepatic cords, are well in line with the earlier reports.[9,28] The histological signs of

recovery of the hepatic architecture in the F+J group, such as the appearance of clusters of oval (pro-generator) cells, hepatoblastic proliferation and their differentiation into hepatocytes, and the rehabilitation of hepatic cords, are clear signs of liver regeneration[29] and thus establish the hepato-curative potentials of JE. These findings are also comparable to earlier reports of the amelioration of toxicity, after exposure in rats, by both seed and leaf extract treatment of carbon tetrachloride (CCl_4) toxicity and by JE treatment of paracetamol toxicity, thus indicating that jambul has a hepato-curative potential.[30-32] The precious antioxidants present in JE may be particularly involved in the rapid hepato-regenerative activity seen in the F+J group.

Although it has been shown that F treatment in rats caused major pathological alterations in the proximal portion of kidney tubules,[33] we did not find any prominent signs of renal histopathology in mice. Nevertheless, the nephronal micrometric results of this study, showing a significant decrease in mean CSAs of the glomeruli and the proximal kidney tubules on F treatment, are consolidating the existing indication that chronic F exposure has caused vascular congestion leading to the shrinkage of the glomeruli and Bowman's capsule in rats.[34] The micrometric data clearly indicate that JE has a ameliorative and rehabilitative effect upon the renal damage caused by F exposure. Based upon these findings it is suggested that chronic F exposure in drinking water (50 ppm F ions) for 10 days or more can slow down performance behaviors by means of induced muscular weakness and neurotoxicity (resulting in the regression of neuromotor coordination). The induced hepato-nephronal pathologies with F exposure may also be contributing towards the regression in neuromuscular performance as these organs bear the main part of the stress of increased metabolite levels and impairment of the ready removal of excretory products. The prominent signs of amelioration, on JE treatment in all the studied parameters, suggest that JE possesses hepato-nephronal and neuromuscular rehabilitative potentials against the multiple organ toxicity that may occur with F exposure.

CONCLUSIONS

Fluoride exposure in mice leads to various hepato-renal histopathologies and alterations in performance behaviors. These toxicities were convincingly ameliorated with JE treatment.

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