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Effect of spinach enriched with zinc and ω-3/Vitamin E treatment against fluoride-induced nephrotoxicity and bone calcification disturbance in rats

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ABSTRACT

Objective: The goal of this study was to evaluate the alone or associated spinach, zinc, and ω-3/Vitamin E capacity to improve the renal function and bone calcification altered by fluoride in Wistar rats. **Methods:** In this study, 36 female Wistar albino rats were used; divided into six groups (n = 6); all groups except the control rats received orally 400 ppm of fluoride as sodium fluoride (NaF) in drinking water during 70 days and then four groups treated in the past 15 days by: Spinach powder, zinc, ω-3/Vitamin E, and spinach+ zinc + ω-3/Vitamin E. Some biochemical and histological markers were analysis. **Results:** Exposure to fluoride altered significantly ($P < 0.05$) the kidney function and structure, calcium status, and induced kidney oxidative stress by decreasing glutathione (56%) and increasing the Malondialdehyde (26%) levels compared to control. Treatments with spinach enriched with zinc and omega 3 modulate calcium levels, kidney oxidative stress status, and attenuate kidney histological disturbance **Conclusion:** Single or interactive treatments administration exhibited a significant normalized effect on bone calcification disturbance, histological alterations, and oxidative stress state in the rats kidney induced by NaF.

Keywords: Bone calcification, NaF, nephrotoxicity, spinach, zinc, ω-3/Vitamin E

INTRODUCTION

Fluoride is an important electronegative anion widely used in the pharmaceutical and agrochemical industries[1] such as fluoridated foodstuffs, different insecticide formulations, and dental products such as mouthwash and toothpaste, and vapors from industries using fluoride-containing compounds.[2] It is well known that fluoride could cause bone damage, such as fluorosis and osteoporosis[3] and the consequences of fluorosis are lesions of the bones and teeth.[4] Excessive fluoride intake can manifest not only as dental and skeletal fluorosis but can also affect soft tissues.[5] Because of the kidney is a major organ involved in the elimination of fluoride from the body, so is consequently susceptible to fluorosis.[6] Chronic kidney disease (CKD) which is recognized as progressively deterioration of renal function has become a growing global health crisis,[7] and receiving increased global attention because of a significant increase in the disease prevalence.[8] The incidence of CKD in Algeria has been estimated as 34 per million people per year. Each year, this amounts to nearly 1000 new patients.[9] Zinc is an essential trace element for most organisms.[10] Low dietary zinc intake may increase the risk of CKD development in individuals with normal renal function.[11] In patients with CKD not receiving yet dialysis, n-3 PUFA may lower the risk of progression to end-stage kidney disease.[12] Higher plasma polyunsaturated omega-3 fatty acid concentrations were associated with slower loss of creatinine clearance.[13] Vitamin E is an essential micronutrient abundantly represented in seeds, edible oils, and fats,[14] this Vitamin is usually associated with moderate and asymptomatic deficiency during renal dysfunction.[15] Since omega-3 fatty acids are sensitive to oxidation, its cosupplementing with Vitamin E could be a suitable strategy to achieve better outcomes.[16] *Spinacia oleracea* is one of the most popular leafy vegetables.[17] Considering fluoride is not under homeostatic control and it is cleared from the plasma within few hours

by the complementary action of calcified tissues and the kidneys[18] and considering spinach contain zinc[19] and ω-3.[20] In the previous studies, we find that spinach is rich in bioactive substances that have several biological effects, but the presence of some acids, such as oxalate, affects the absorption of some minerals such as zinc, and because zinc is known for its biologically active role, as well as omega and Vitamin E, which have anti-inflammatory and anti-oxidants activities, so the main and new idea in our choice of treatment in this study is to be an integrative treatment between several proven elements such as zinc, omega, and Vitamin E supported by the natural treatment of spinach, which contains several bioactive molecules that have nephroprotective activity and modulating impact on bone calcification induced by NaF in rats.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium fluoride and all of the chemicals and reagents was analytical grade and provided from Merck, 134 (Darmstadt, Germany).

Experimental Animals

Thirty-six healthy female Wistar rats at the age of 8 weeks old with a weight of 180–230 g were purchased from the Institute Pasteur of Algiers. Rats were acclimated to the laboratory conditions for 2 weeks and housed in an animal room of molecular and cellular biology department of El-Oued University, Algeria, with a temperature of 23 ± 2°C and a relative humidity 65.3%. The rats were adapted to an inverse 12:12 h light/dark cycle. All experimental procedures employed, also rat care and handling, were in accordance with guidelines provided by the ethics committee (17EC/DCMB/FNSL/EU2020) of the department of cellular and molecular biology, Faculty of Natural Sciences and Life, El-Oued University.

Experimental Design

Animals were divided into six groups (n = 6).

- Control: Received standard diet and water.
- NaF exposed rats: Received standard diet and 400 ppm of sodium fluoride (NaF) in their drinking water.
- NaF exposed rats+ spinach: Received standard diet with spinach powder (17g/kg feed).
- NaF exposed rats+ ω-3/vitamin E: Received standard diet (1.825g Eicosapentaenoic acid (EPA)+1.21g docosahexaenoic acid (DHA)+ 83.3 mg Vitamin E/Kg of feed).
- NaF exposed rats+ zinc: Received standard rat food with zinc (0,230g/kg feed) as $znSO_4$, $7H_2O$.
- NaF exposed rats+ (spinach, ω-3/vitamin E, zinc): Received standard rat food with the same previously doses.
- All treated groups except control received NaF in their drinking water.

ω-3/Vitamin E as (1.825g (EPA)+1.21g (DHA)+ 83.3 mg Vitamin E,[21] zinc (0.231g/kg feed) as ZnSO4 7H2O,[22] and spinach (the dose was chosen according to its traditional use) were added to the animals' in the appropriate experimental feed for 21 days.

Blood Sampling and Tissue Collection

At the end of each treatment, and after 12 h of fasting, animals were dissected; blood sampling was performed during the decapitation. The serum was prepared by centrifugation, for 10 min at 3000 revolutions/min and utilized for biochemical analysis assays; kidneys and bone were rapidly excised, weighed, and stored at −20°C for oxidative stress parameters, calcium level, and histological analysis.

Qualitative and Quantitative Analysis

According to method described by Altemimi *et al*.,[21] six assays were used to identify phytochemical compounds of alkaloids, saponins, glycosides, tannins, phenols, and flavonoids in *S. oleraceae* extract. Phenols are determined by the Folin-Ciocalteu method.[24] Total flavonoid content of spinach leaves extract is carried out by the method described by Ahn *et al*.[25]

DPPH Radical Scavenging Activity

DPPH scavenging assay was performed on the basis of reduction of DPPH recorded at 517 nm. Ascorbic acid was used as well-known standard antioxidant substances. Inhibition percentage (IP) of DPPH was calculated according to**:**

$$(IP) = [(A_{control} - A_{sample}) \times 100]/A_{control}$$

$A_{control}$ is the absorbance of the control (containing all reagent except the sample), and A_{sample} is the absorbance of the sample.

IC_{50} value was determined by linear regression analysis of IP versus concentration and represents the concentration of extract that gives 50% of reduction in DPPH absorbance (free radicals level).[26]

Calcium Spinach and Bone Levels

Dried spinach and bone were heated in silica crucibles at 600°C for 6 h and the ash was dissolved in hot nitric acid for calcium using a Flame photometer (Jenway PFP7). The calcium standards were prepared from a 1 mg/mL calcium carbonate.

Biochemical Markers Levels

Creatinine, urea, and uric acid were determined using biochemical auto-analyzer. Serum sodium and potassium levels were determined using electrolyte auto-analyzer (Easylyte PLUS Na/K/CL de Medica).

Oxidative Stress Biomarkers

About 1 g of each kidney was homogenized in 9 mL (10% (w/v)) of buffer solution of Tris buffer saline (pH = 7.4). Homogenates were centrifuged at 5000 revolutions/min for 15 min at 4°C, and the obtained supernatant was used for the determination of antioxidant activity. The concentrations of reduced glutathione (GSH) were measured in the supernatant according to the methods described by Weckbecker and Cory.[27] The complex formed between GSH

and 5,5'dithiodis-2-nitrobenzoic acid releases thionitrobenzoic acid which has an absorbance at 412 nm. Total GSH content was expressed as nmol GSH/mg prot. Malondialdehyde (MDA) was measured according to Sastre *et al*.[28] The reaction results in the formation of a pink complex between two molecules of thiobarbituric acid which can, therefore, be measured by Absorption spectrophotometry at 532 nm and the level of MDA in liver was expressed as nmol/mg protein.

Histopathological Sections

After sacrifice, one kidney from each rat group was removed immediately and stored in formalin solution (10%). Sections 5 μm thick were cut, dehydrated in ascending graded series of ethanol, cleaned with toluene, immersed in paraffin, and colored with hematoxylin and eosin. Histopathological evaluation was performed with a light microscope.

Statistical Analysis

Our statistical study is carried out by the software program (Minitab 17) using Student's t test to compare means among our different experimental groups; the results are in the form of mean and standard error. Differences were considered statically significant at $P < 0.05$.

RESULTS

Phytochemical Study

The results of the qualitative analysis of spinach show that the extract of the plant contains important secondary metabolites such as phenols, flavonoids, saponins, and tannins. The results also show that the plant lacks of alkaloids and glycosides as shown in Table 1.

The results obtained through the quantitative study [Table 2] showed that the spinach plant is rich in total phenols, especially flavonoids, and also calcium analysis shows that the plant is an important source of calcium in significant quantities. On the other hand, the results show that the spinach plant has a significant antioxidant capacity, and this is through the DPPH analysis.

Body and Kidney Relative Weights

As revealed in Table 3, there was very highly significant elevation ($P < 0.001$) in the relative weight of kidney in the fluoride exposed group compared to control rats. In Figure 1, we only demonstrated the effect of fluorine through the size of the kidney exposed to it compared to the normal size in the control rats. A significant amelioration ($P < 0.05$) was observed in the kidneys relative weights after the alone spinach administration while this amelioration was highly significant ($P < 0.01$) if the rats were treated by both alone ω-3/Vitamin E and alone zinc or if the rats treated by the combination of all elements (spinach+ ω-3/Vitamin E+ zinc) compared with fluoride exposed group. However, no significant change of weight in Sp and zinc group compared to control and association group compared to NaF group. The spinach was the less treatment decreases the relative kidney weight while the ω-3/Vitamin E revealed the better ameliorated treatment.

Table 1: Phytochemical essays of *Spinacia oleraceae* extract

Compounds	Spinach extract
Alkaloids	–
Saponins	+
Glycosides	–
Tannins	+
Phenols	+
Flavonoids	+

Table 2: Total phenols, flavonoids, and calcium concentrations in *Spinacia oleraceae*

Compounds	*Spinacia oleraceae*
Phenols (mg of GAE/g of crude extract)	42±0.658
Flavonoids (mg of QE/g of crude extract)	5.88±0.415
Calcium (mg/g of spinach powder)	0.328±0.02
DPPH IC_{50} (μg/ml)	6.23±0.14
DPPH IC_{50} *(Ascorbic acid) (μg/ml)*	3.41±0.063

Figure 1: Morphological appearance of normal kidney (at the right) and fluoride exposed kidney rat (at the left)

Biochemical Parameters

Creatinine, urea, and uric acid as renal function biomarker revealed significant increase ($P < 0.05$) in fluoride exposed group when compared with the control. No significant improving was observed in neither experimental group which showed an irreversible kidney dysfunction. Both sodium and potassium levels showed a significant ($P < 0.05$) elevation in fluoride exposed rats compared to control; continuing in the sodium level increase during different treatments compared to control and fluoride exposed groups; just the spinach group did not change compared to fluoride exposed group, while in the potassium, we observe a slight restoration of its level compared with the fluoride exposed group just in ω-3/Vitamin E ameliorate significantly ($P < 0.05$) the potassium level compared to control. A high significant increase ($P < 0.01$) in serum calcium level concomitantly with significant decrease ($P < 0.05$) of that in bone in fluoride exposed rats compared with control; continuing in the serum calcium level increase during the alone spinach administration with a restoration of

bone calcium level in that group compared to fluoride exposed group. Both alone zinc and ω-3/Vitamin E also the combination of all treatments decreased high significantly ($P < 0.01$) the serum calcium levels compared to fluoride exposed rats but in the bone just the zinc group, the calcium level was raised significantly ($P < 0.05$) compared to control and fluoride exposed groups whereas the alone ω-3/Vitamin E and the combination of all treatments showed a partial restoration compared to fluoride exposed group ($P > 0.05$) [Table 4].

Oxidative Stress Parameters

The present study revealed high significant ($P < 0.01$) increased levels of MDA simultaneously with very high significant ($P < 0.001$) decreased levels of GSH in the kidney of fluoride exposed rats compared with control rat. In rats treated by spinach, ω-3/Vitamin E, zinc, and combination of all these components, we observe that oxidant/antioxidant balance was significantly regulated; this last has been demonstrated by the decreasing on MDA level and non-enzymatic (GSH) antioxidant defense systems [Table 5].

Histological Analysis

The morphologic characteristics of the kidneys were assessed macroscopically. There was no difference between the groups in relation to the macroscopic features; just the kidney size of fluoride rats group appears more big [Figure 1]. In histopathology appearance, the kidneys of the control group were normal and show glomeruli and a compact tissue appearance [Figure 2.a1.a2]. The tissue of kidney fluoride exposed rats shows the presence of dissolved cells due to cell necrosis, and also seen are widespread infiltrations by inflammatory cells [Figure 2.b1.b4]. Severe edema, tubular degeneration, and tubular dilatation were seen [Figure 2.b3] also hemorrhage was massive in that group [Figure 2.b2]. Glomerular atrophy and big Bowman's space were observed [Figure 2.b3]. We observe noticeable improvements in tissue architecture as there were more visible glomeruli and fewer inflammatory cells and effectively reduced the kidney tissue damage after spinach, ω-3/Vitamin E, and zinc supplementation [Figure 2c-f]. Cellular damage was higher in fluoride exposed group than in the treated groups. In fact, zinc and associated treatment are the totally reversed the kidney damage induced by fluoride in rats. The histopathological changes were scored in Table 6.

DISCUSSION

In our present study, fluoride induces renal function alteration and kidney hypertrophy (nephromegaly). Kidney function biomarkers of all experimental groups revealed a renal

Table 3: Changes in body and relative kidney weights of control and experimental rats groups

	Control (*n*=6)	NaF (*n*=6)	NaF+Sp (*n*=6)	NaF+ω-3V (*n*=6)	NaF+Zn (*n*=6)	NaF+Sp+Zn+ω-3V (*n*=6)
Initial body weight (g)	241.2±12.7	198.7±25.2	191±9.71	209.5±10.7	207.33±6.17	228.3±20.2
Final body weight (g)	257.7±7.20	188.7±17.2*	187.7±8.21***^{NS}	177.63±1.53***^{b}	190.47±1.72*^{NS}	226.9±15.6^{NSa}
Relative Kidney Weight	0. 24±0.032	0. 84±0.036***	0. 58±0.053**^{a}	0. 35±0.033*^{b}	0. 53±0.044**^{b}	0. 55±0.073**^{b}

Means±SE from animals in each group. Comparison with the control group: *P<0.05, **P<0.01, ***P<0.001.
Comparison with the fluoride exposed group: ^{a}P<0.05, ^{b}P<0.001, ^{c}P<0.001

Table 4: Renal function biomarkers, bone, and serum calcium and electrolytes levels of control and experimental rats groups

	Control (*n*=6)	NaF (*n*=6)	NaF+Sp (*n*=6)	NaF+ω-3V (*n*=6)	NaF+Zn (*n*=6)	NaF+Sp+Zn+ω-3V (*n*=6)
Creatinin (mg/L)	10.92±0.278	19.35±1.86*	19.33±2.56*	19.50±2.46*	21.65±1.41**	24.16±0.97***^{b}
Urea (dg/L)	0.78±0.01	1.03±0.058**	1.17±0.059 ***^{a}	0.96±0.031**	1.0±0.026**	1.52±0.034***c
uric acid (mg/L)	11.50±1.28	19.5±1.32**	22.75±0.859***^{a}	20.50±1.32**	17±0.837**a	10.62±0.99^{b}
Serum Na^{+} (mmol/L)	140.33±1.11	143±0.57*	144.95±0.95*	154.60±0.40***^{c}	149.03±0.59**^{b}	149.26±0.99*^{b}
Serum K^{+} (mmol/L)	7.438±0.247	9.94±0.566*	8.60±0.50	8.28±0.40^{a}	8.527±0.784	8.435±0.984
Serum Ca^{+2} (mg/L)	100.8±1.36	130±4.95**	143±3.13***^{a}	116±2.83**^{b}	112.35±3.5*^{b}	112±3.58*^{b}
Bone Ca^{+2} (mg/g tissues)	2.252±0.153	1.706±0.117*	2.439±0.104 ^{a}	2.104±0.115	3.255±0.215*^{a}	2.023±0.102

Means±SE from animals in each group. Comparison with the control group: *P<0.05, **P<0.01, ***P<0.001.
Comparison with the fluoride exposed group: ^{a}P<0.05, ^{b}P<0.001, ^{c}P<0.001

Table 5: Renal MDA and GSH levels of control and experimental rats groups

	Control (*n*=6)	NaF (*n*=6)	NaF+Sp (*n*=6)	NaF+ω-3V (*n*=6)	NaF+Zn (*n*=6)	NaF+Sp+Zn+ω-3V (*n*=6)
MDA (µmol/g tissue)	2.821±0.128	3.81±0.23**	2.35±0.17^{a}	2.84±0.16^{a}	2.45±0.15^{a}	2.14±0.37^{a}
GSH (nmol/g tissue)	0.754±0.026	0.34±0.023***	0.59±0.025**^{c}	0.74±0.008^{NSc}	0.52±0.020***^{c}	0.60±0.03**^{c}

Means±SE from animals in each group. Comparison with the control group: *P<0.05, **P<0.01, ***P<0.001.
Comparison with the fluoride exposed group: ^{a}P<0.05, ^{b}P<0.001, ^{c}P<0.001

Table 6: Grading of the histopathological changes in kidney sections of control, hypothyroidism, and treated rats

Alterations	Experimental groups					
	Control	NaF	NaF+Sp	NaF+ω-3/VitE	NaF+Zn	NaF+Sp+Zn+ω-3/VitE
Necrosis	-	+++	+	+	-	+
Edema	-	+++	+	++	-	+
Hemorrhage	-	+++	++	+	-	+
Interstitial cell infiltration	-	+++	+	-	-	+
Tubular dilatation	-	+++	-	-	+	+
Inflammation	-	+++	++	+	-	+
Glomerular hypertrophy	-	-	+	-	-	-
Glomerular atrophy	-	++	-	-	-	+
Bowman's space	+	+++	+	+	+	++

None (-), moderate (+), and severe (++)

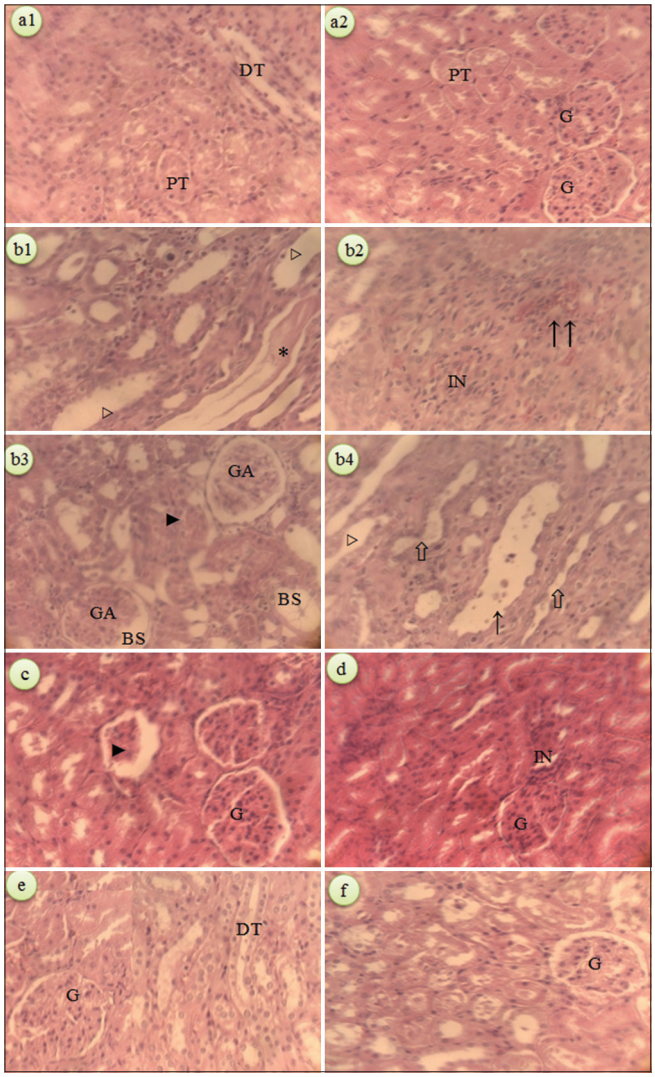

Figure 2: Histopathological section of kidney, control rats (a1 and a2), fluoride exposed rats (b1, b2, b3, b4), and treated rats; (c) NaF+ Spinach. (d) NaF+ ω-3/Vitamin E. (e) NaF + Zinc. (f) NaF +(Spinach, ω-3/Vitamin E, Zinc) (×400). (PT) proximal tubules, (DT) distal tubules, (g) glomerulus, Necrosis (►), edema (*), interstitial cell infiltration (↑), and tubular dilatation (⍰), tubular degeneration (⇧), hemorrhage (↑↑), glomerular atrophy (GA), Bowman's space (BS), and inflammation (IN) and no apparent pathology in control rats (×400).

dysfunction translated by high significant increase of creatinine level confirmed by that of urea and uric acid. The comparison with fluoride exposed group showed that no treated group improves the state of renal dysfunction, which means that the renal dysfunction was irreversible. Kidney hypertrophy in fluoride exposed rats probably interpreted by the appearance of hydronephrosis which occurs when the renal collecting system of one or both kidneys becomes dilated from the obstruction of urine outflow.[29] In our experimentation, the loss of renal function may be caused by calculi because fluoride *in vivo* may behave as a promoter of urinary stone formation by excretion of insoluble calcium fluoride and increasing oxalate excretion[30] and our findings revealed that serum calcium in rats exposed to fluoride significantly higher compared to control rats; so the increase of the relative kidney weight may be the result of calcium fluoride formed stone (calculi) that enhanced during renal plasma flow rate decrease in this situation. Furthermore, the prolonged on-set of hyperuricemia is associated with the development of kidney stones and renal failure;[31] thus in addition of reduction in glomerular filtration rate, the dysfunction itself can be caused also by the accumulation of kidney stones. Both excess and insufficient zinc promotes stone formation.[32] Spinach leaves contained of organic acids (malate, succinic acid, ethanedioic acid, 2-oxoglutarate, and citrate),[33] also it is the main sources of chlorophylls,[34] this last when degraded, chlorophyll derivatives are formed through the loss magnesium ion of chlorophyll[35] so the spinach is a rich source of magnesium. Magnesium is also a crystallization inhibitor. It reduces urine calcium oxalate super saturation, thus inhibiting growth and aggregation of calcium oxalate crystals. Oxalate content is high in spinach, water spinach and other vegetables or plants;[36] therefore, the less improver effect of spinach may be refer to that it contain the main important crystallization inhibitor and the main important element of stone formation, and this can be the explanation of the significant increase of the relative kidney weight compared with the control group in alone zinc, alone spinach or coadministration of zinc, spinach, and omega 3/Vitamin E although just the Omega 3/Vitamin E administrated group revealed the better amelioration in relative kidney weight among all our treated experimental groups. Supplementation of Vitamin E keeps the cellular membranes intact and protects the kidneys from damage caused by oxidative stress, thereby curtailing the concentration of calcium, oxalate, and uric acid

and decreasing the supersaturation of urine thence crystal retention. Free radicals mediated injury to renal tubular cells is considered as one of the prerequisites for crystal retention.[37]

The present study revealed increased of MDA levels, simultaneously with decreased levels of GSH in the kidney of fluoride exposed rats compared with control rats which confirm the disturbance of oxidant/antioxidant balance. Lipid peroxidation (the marker of its extent is the MDA level) a degenerative pathway of the membrane components mediated through the free radicals produced in the cell, is a hallmark feature of oxidative stress.[38] In our study, we supplemented the rats by fluoride which the fluoride is a strongest oxidant because of high electronegativity.[39] Fluoride causes an oxidative stress in the kidney[40] as the kidney the main organ of fluoride eliminating. In rats treated by spinach, omega 3/Vitamin E, zinc, and combination of all these components, we observed that oxidant/antioxidant balance was regulated. Depending on Giardi *et al*., 2013 who showed that the protective effect of *S. oleraceae* L. against mice radiation-induced oxidative stress by its constituents, including b-carotene, lutein, Zeaxanthine, flavonoids, Vitamin C, and p-coumaric acid.[41] Manganese and zinc contents are present in maximum amounts in Spinach.[42] In contrast, zinc supplementation decrease MDA and raised GSH kidney levels. These changes confirm an efficacious defense of the zinc against oxidative stress.[43] Zinc is also necessary to stimulate defense against reactive species oxygen and H_2O_2 that induce apoptosis and superoxide dismutase (SOD) activity.[44] Furthermore, the previous studies have demonstrated that combined omega-3 fatty acids and Vitamin E intake might have strong synergistic effects on related markers of glucose and fatty acids metabolism which, in turn, may act more effectively on biomarkers of oxidative stress and inflammation.[45] Both sodium and potassium levels increase in fluoride exposed rats may be refer to loss of kidney function, because in patients with advanced CKD, the progressive decline in kidney function contributes to the development of hyperkalemia[46] and hypernatremia.[47] Our previous studies[48,49] demonstrated that alteration of thyroid gland cause electrolytes levels perturbation, also the study done by Mogulkoc *et al.*[50] who indicated electrolyte changes in hypothyroidism. Triiodothyronine and L-thyroxine participate in the regulation of liquid-electrolyte balance. We observed that there is an inverse relationship between serum and bone calcium levels. Bone, as in the case of lead, acts as a natural sink for fluoride; F is not irreversibly bound to bone; it is released after cessation of excess F uptake[51] so the fluoride bound in the bone causing release of calcium which confirm the elevation of serum calcium level. Our findings indicate that spinach is rich of calcium, which can be explain the increase of serum and bone calcium levels in spinach treated rats. According to an *In vitro* study results obtained by Boeyens *et al.*[52] who revealed the inhibitory effects of DHA on osteoclast formation, we can explain the effect of omega 3 administration. *In vivo* level supplementation with omega 3 PUFAs not only suppresses bone resorption but also promotes new bone formation of rats by promoting osteoblastogenesis in the lesion.[53] Both alone zinc or its combination with spinach and ω-3/Vitamin E present the better improver agent in serum and bone calcium levels; this may refers to the reverse relationship between calcium and zinc; because one of the most common trace metal imbalances is elevated calcium and depressed zinc or the inverse.[54] According to the results of the present study, NaF exposure produces histological damage in the kidney including glomerular necrosis, tubular dilation, and inflammation. These alterations may be due to the excessive production of free radicals and as a result of lipid peroxidation induced by Fluor.[55] Treatment with *S. oleracea* and/or zinc/omega intoxicated rats has been able to regenerate the structure of the kidney. Our results suggest that spinach could reduce kidney lesions induced by NaF. The biochemical and oxidative stress parameters are also correlated with the histological study. This can be attributed to the anti-radical effect of spinach. Our results prove that spinach is rich in phenols, flavonoids, and tannins, which are secondary metabolites that have biological anti-oxidant and anti-inflammatory effects.[56] These plants reduced the oxidative stress caused by fluore, allowing the reduction of histological alterations and restoration of the normal physiological state of the body. Our results prove that spinach is rich in phenols, flavonoids, and tannins, which are secondary metabolites that have biological anti-oxidant and anti-inflammatory effects.[57] On the other hand, zinc is an essential element that has antioxidant functions by being a cofactor for several antioxidant enzymes such as SOD, which helps to reduce kidney tissue lesion.[58]

CONCLUSION

Single and associated treatments present a significant normalized effect on bone calcification disturbance and beneficial effects of on histological kidney alterations and oxidative stress caused by fluoride. Further studies are needed to understand the molecular basis behind these treatments and the necessary time and dose to reverse totally the fluoride induced nephrotoxicity.

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