AN INVESTIGATION OF THE PROTECTIVE EFFECTS OF RESVERATROL ON SOME BIOCHEMICAL PARAMETERS AND HISTOPATHOLOGICAL FINDINGS IN EXPERIMENTALLY-INDUCED CHRONIC FLUROSIS IN RATS

Serkan Yildirim,[a] Gokhan Oto,[b] Bahat Comba,[c] Suat Ekin,[d] D Ali Cinar[c,*]

Erzurum and Van, Turkey

ABSTRACT: This study was carried out to determine the protective effects of resveratrol on some biochemical parameters and histopathological findings in experimentally-induced chronic fluorosis in rats. Forty male Wistar albino rats, weighing 250–300 g, were randomly divided into four groups of 10 rats and treated for 12 weeks as follows: (i) control group (drinking water=tap water), (ii) resveratrol group (drinking water=50 mg resveratrol/L), (iii) NaF group (drinking water=10 mg NaF/L), and (iv) NaF+resveratrol group (drinking water=10 mg NaF + 50 mg resveratrol/L). The animals were sacrificed at the end of the 12 weeks. The hematological values of the control, resveratrol, NaF, and NaF+resveratrol groups were, respectively: RED BLOOD CELLS: 6.99±0.35, 6.77±0.41, 6.60±0.57, and 6.84±0.65 $\times 10^6$/mm^3; HEMATOCRIT VALUE: 40.93±2.47, 43.54±3.38, 40.67±3.12, and 40.73±5.21 %; HEMOGLOBIN CONCENTRATION: 13.74±0.41, 14.62±0.37, 13.00±0.49, and 13.38±0.52 g/dL; PLATELETS: 369.85±65.73, 383.67±71.15, 291.25±66.51, and 351.83±71.23 $\times 10^3$/mm^3; VITAMIN E: 1.042±0.045, 1.060±0.088, 0.977±0.070, and 0.998±0.060 µmol/L; VITAMIN A: 3.141±0.107, 3.071±0.134, 2.555±0.093, and 2.90±0.131 µmol/L; VITAMIN D: 0.653±0.043, 0.621±0.039, 0.419±0.039, and 0.582±0.046 µmol/L, and VITAMIN K: 0.778±0.047, 0.756±0.068, 0.706±0.052, and 0.725±0.053 µmol/L. The hematocrit hemoglobin levels in the resveratrol group were significantly increased ($p<0.05$) compared to the other groups. The platelet counts, vitamin A, and vitamin D levels were significantly lower ($p<0.05$) in the NaF group than in the other groups. The histopathological findings were: (i) in the control and resveratrol groups, the liver and bone tissue were found to have a normal histological structure, (ii) in the NaF group, hydropic degeneration and colangiohepatitis were detected in the liver, (iii) in the NaF+ resveratrol group, liver degeneration and colangiohepatitis were not found in the liver, (iv) in the NaF group, thinning of the bone tissue trabeculae and a significant decrease in the cellular density of the epiphyseal growth plate were observed, (v) in the NaF+resveratrol group, a slight degree of thinning was detected in the bone trabeculae and the epiphyseal plate. It was concluded that resveratrol has protective effects on some biochemical parameters and histopathological findings in chronic fluorosis.

Key words: Blood parameters; Chronic fluorosis; Histopathological findings; Rat; Resveratrol.

INTRODUCTION

Fluorine, the lightest of the halogens, is the most reactive of the elements and does not occur in the free state in nature but occurs widely in the earth's crust and in nature in combination with other elements and in minerals as the fluoride ion (F). F may accumulate in bones and teeth and have important effects on cellular activities but is not an essential trace element in mammals or necessary for the development of healthy teeth and bones.[1-3] A small number of plants synthesize organofluorine

[a]Department of Pathology, Faculty of Veterinary Medicine, Ataturk University, Erzurum, Turkey; [b]Department of Pharmacology, Faculty of Medicine, Yuzuncu Yil University, Van, Turkey; [c]Department of Physiology, Faculty of Veterinary Medicine, Yuzuncu Yil University, Van, Turkey; [d]Department of Chemistry, Faculty of Science Yuzuncu Yil University, Van, Turkey; *For correspondence: Professor Dr D Ali Cinar, Department of Physiology, Faculty of Veterinary Medicine, Ataturk University, Erzurum, Turkey; E-mail: alicinar25@hotmail.com, alicinar25@mynet.com

poisons to deter herbivores. Some foods and water supplies contain fluoride.[4] F has been added to drinking water (water fluoridation) and used topically in some countries to protect teeth from decay.[1,2,5]

High concentrations of F are noxious in the environment and can affect the health of both humans and animals. F toxicity in humans and animals may be both acute and chronic (fluorosis). Volcanic regions are usually rich in fluoride, and chronic fluoride toxicity or endemic fluorosis is often present in such areas.[3,4,6] In recent years, many researchers[7-12] have realized that F accumulates not only in bones and teeth[10] but also, to a lesser degree, in soft tissues,[8] especially the cardiovascular system, brain[9], liver[7], kidneys[12], and spinal cord.[9] F can rapidly cross certain cell membranes[6] and is distributed in skeletal and cardiac muscle, liver, skin, and erythrocytes.[7-12]

A daily intake of 0.5–1.7 mg F /kg body weight (bw), in the form of sodium fluoride may cause various disorders, e.g., dental lesions,[7,13] thyroid dysfunction and hypothyroidism,[14-16] sinusoidal bradycardia,[17,18] stunted growth, hyperparathyroidism,[13] interference with mineral metabolism,[13,19] mechanical neurological complications (radiculopathy, myelopathy)[8], and low milk production in livestock.[1,12]

Resveratrol is a compound that has attracted much attention in the medical and pharmaceutical sectors in recent years. Resveratrol is a phytoalexin which is found in foods including grapes, plums, cranberries, and peanuts. Resveratrol is a strong antioxidant and some have considered that it can retard aging. Resveratrol helps prevent cell damage caused by free radicals by virtue of its strong antioxidant properties, as well as by inhibiting cell death.[20]

The effects of chronic fluorosis on different mechanisms have been examined,[21-25] but there has been only limited study, in rats with chronic fluorosis, of the protective effects of resveratrol on certain vitamins, blood parameters, and liver and bone histopathology. Therefore, the objective of the present study was to investigate the protective effects of resveratrol in experimentally-induced chronic fluorosis in rats on certain biochemical parameters and histopathological findings

MATERIALS AND METHODS

In this study, 40 male Wistar albino rats, weighing 250–300 g, were randomly divided into four groups with one control and three experimental groups (n = 10 per group). Animals were housed in a well-ventilated and air-conditioned area provided with independently adjustable light–dark cycle (12 hr light/12 hr dark cycle) and temperature regulation systems. Temperature was maintained at 22±2ºC and humidity was kept at 45–70 %. The rooms and animal cages were cleaned daily and the animals were provided with fresh food and water *ad libitum* on a daily basis.

The control group was administered tap water for 12 weeks. The resveratrol group was administered drinking water with 50 mg resveratrol/L for 12 weeks,[26] the NaF group was administered drinking water 10 mg NaF/L for 12 weeks,[27] and the NaF+resveratrol group was administered drinking water with 10 mg NaF + 50 mg resveratrol/L for 12 weeks.

The experimental protocol was approved by the Committee on the Ethics of Animal Experiments at Yuzuncu Yil University (Permit Number: 2013/-02).

The blood parameters were determined in whole blood using rat veterinary practice and a blood cell counter (Abocus Junior Vet-5, Austria). The statistical analyses of the blood parameters are presented as mean±standard deviation (mean±SD). SPSS version 20 was used for statistical analysis. ANOVA and DUNCAN tests were used for comparison between groups.

Vitamin A, D, E, and K analysis: α-tocopherol, retinol, phylloquinone, and cholecalciferol in serum were extracted as follows: serum (100 µL) was deproteinized by adding ethanol (100 µL) (containing 0.025 % BHT) (Su et al.). The sample was extracted twice with n-hexane (600 µL). The sample was vortex mixed and centrifuged at 8000 rev/min for 10 min. Part (500 µL) of the hexane layer was extracted and evaporated to dryness under a nitrogen stream at 37°C. The residue was dissolved in tetrahydrofuran (50 µL) and methanol was added (150 µL). The sample was vortexed for 1 min and then 100 µL samples were autosampled using amber glass vials. The mobile phase of a methanol-THF mixture (80:20, v/v) was used. Pump was set at a flow rate of 1.5 mL/min. The chromatographic analysis was performed at 40ºC with isocratic elution. The chromatogram was monitored with photodiode array detector (PDA) array detection at 290, 325, 265, 248 nm (α-tocopherol, retinol, cholecalciferol, and phylloquinone, respectively).[28]

Histopathological examination: Animals were sacrificed at the end of the 12th week of the experimental period by decapitation and liver and bone were quickly removed and processed for histopathology. Liver and bone sections were fixed in 10% neutral buffered formaldehyde for 48 hr. Bone tissues were decalcified via Osteosoft® (Merck, HC313331, Germany) and washed under tap water overnight. Following routine tissue preparation procedures, tissue samples were dehydrated through a graded series of alcohol and chloroform, and embedded in paraffin blocks. Liver tissues were buried in paraffin blocks after the routine tissue procedure. Paraffin serial sections were cut at a thickness of 5 µm and mounted on glass slides. Bone and teeth were sectioned at a 5 µm thickness, stained with H&E, and examined under a light microscope (Olympus BX51 and DP25 digital camera, Japan). The extent of staining was scored as – (negative), + (slight), ++ (moderate), and +++ (severe) according to the histopathological findings.

RESULTS

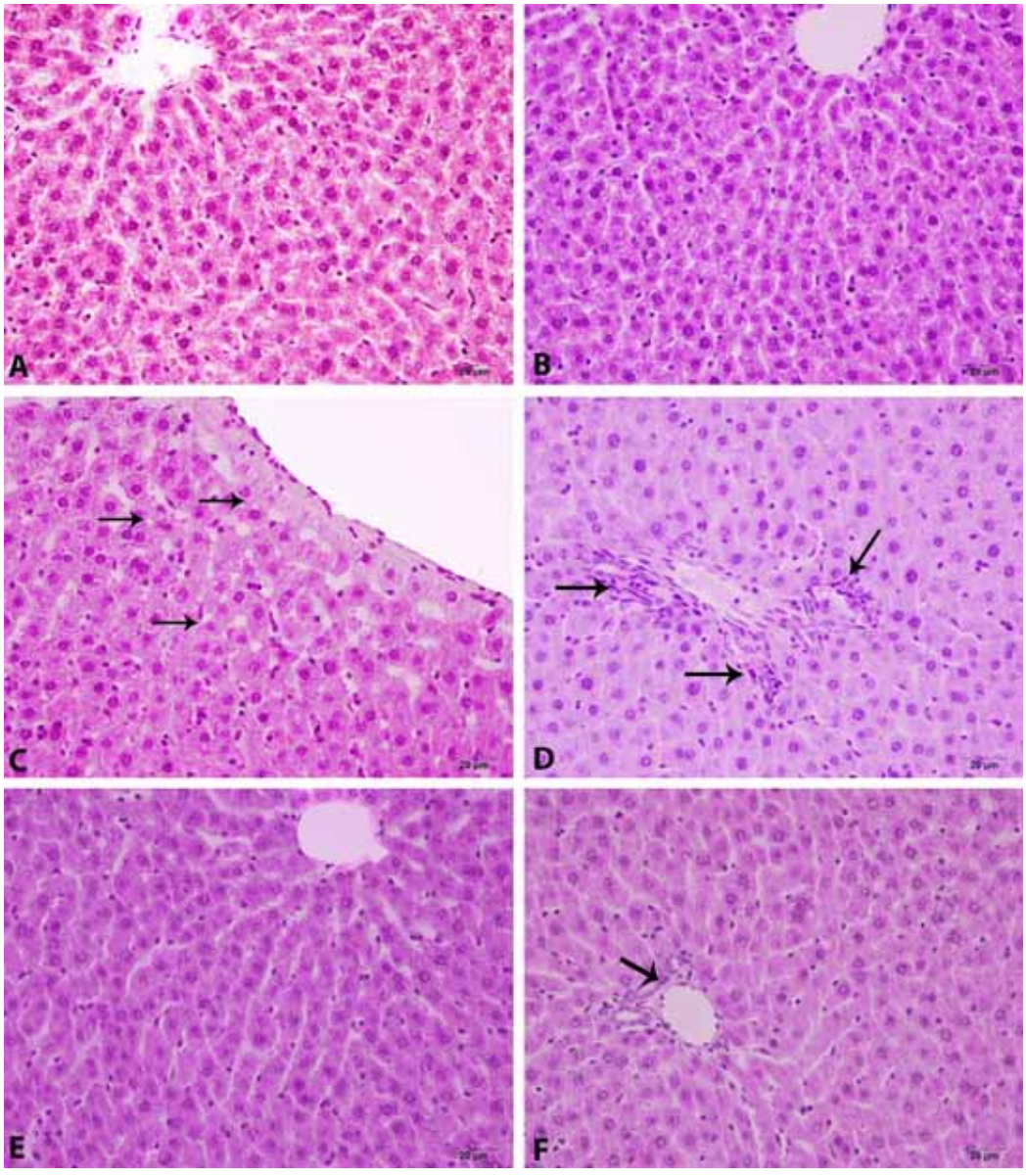
The results of the blood parameters and histopathological findings in the groups are presented in Table 1. As seen in Table 1, the hematocrit value and the hemoglobin level were increased in the resveratrol group compared to the control group. The platelet counts and the vitamin A and D levels were significantly lower in the NaF group than in the control group ($p<0.05$).

Table. Some blood parameters and vitamin levels in the groups (values are mean±SD, n=10, RBC=red blood cell count, HCT=hematocrit value, Hb=hemoglobin concentration, PLT=platelet count)

Blood parameter	Groups			
	Control group (drinking water=tap water)	Resveratrol group (drinking water with 50 mg resveratrol/L)	NaF group (drinking water with 10 mg NaF/L)	NaF+Resveratrol group (drinking water with 10 mg NaF/L + 50 mg resveratrol/L)
RBC ($\times 10^6/mm^3$)	6.99±0.35	6.77±0.41	6.60±0.57	6.84±0.65
HCT (%)	40.93±2.47[a]	43.54±3.38[b]	40.67±3.12[a]	40.73±5.21[b]
Hb (g/dL)	13.74±0.41[b]	14.62±0.37[c]	13.00±0.49[b]	13.38±0.52[b]
PLT ($10^3/mm^3$)	369.85±65.73[d]	383.67±71.15[d]	291.25±66.51[e]	351.83±71.23[d]
Vitamin E (µmol/L)	1.042±0.045	1.060±0.088	0.977±0.070	0.998±0.060
Vitamin A (µmol/L)	3.141±0.107[f]	3.071±0.134	2.555±0.093[g]	2.904±0.131
Vitamin D (µmol/L)	0.653±0.043[h]	0.621±0.039	0.419±0.039[i]	0.582±0.046[i]
Vitamin K (µmol/L)	0.778±0.047	0.756±0.068	0.706±0.052	0.725±0.053

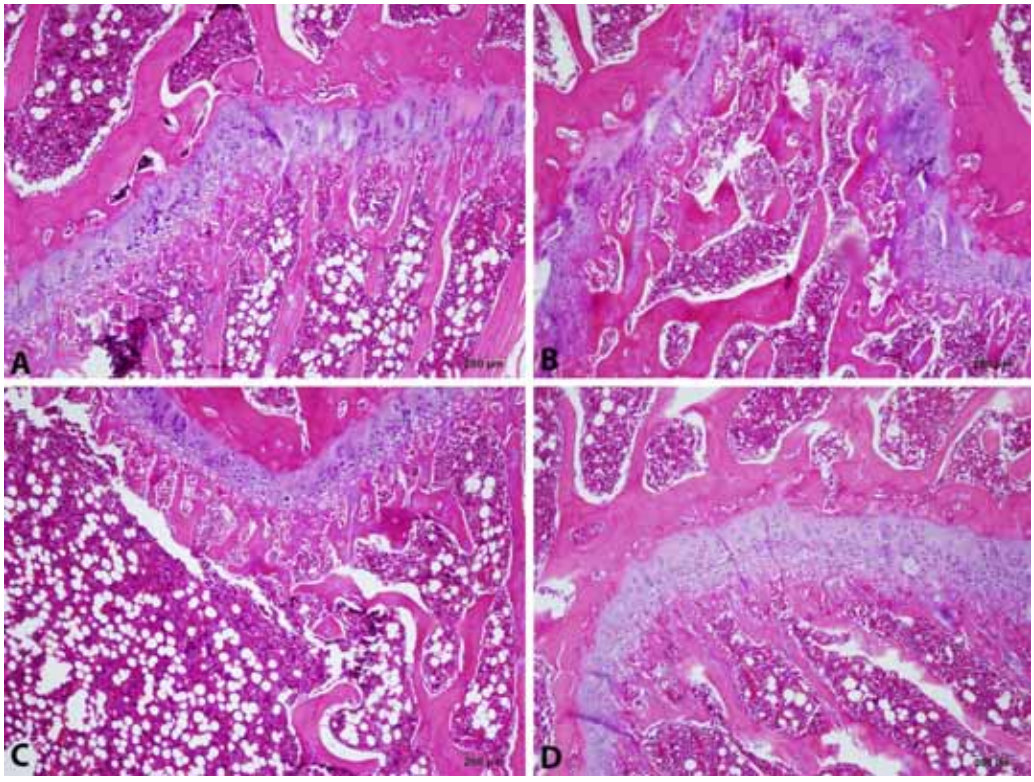
The differences among the groups with different letters in the same line were significant ($p<0.05$).

The livers of the rats in the control and resveratrol groups had a normal (–) histological structure (Figures 1 A and 1B). In the NaF group, degeneration of hepatocytes with fat vacuoles (+) were found in some hepatocytes in the central region of the liver (Figure 1 C) and colangiohepatitis, bile duct proliferation, and congestion (++) were found in the portal region (Figure 1 D). In the NaF + resveratrol group, the central region was normal (–) (Figure 1 E), while the portal region had low levels of bile duct proliferation and no mononuclear cells (–) (Figure 1 F). [Scoring of histopathological finding: – (negative), + (slight), ++ (moderate), and +++ (severe)].



Figures 1A-1F. A: control group showing a normal (–) histological appearance of the liver, **B:** resveratrol group showing a normal (–) histologic appearance of the liver, **C:** NaF group showing hydropic degeneration in some of the hepatocytes (+) of the central region (arrows), **D:** NaF group showing mononuclear cell infiltration (++) of the portal region (arrows), **E:** NaF + resveratrol group showing a normal (–) histologic appearance of the central region, and **F:** NaF + resveratrol group showing a low level of bile duct proliferation (+) in the portal region (arrow). H & E staining, bar=20 µm. Scoring of histopathological findings: – (negative), + (slight), ++ (moderate), and +++ (severe).

Examination of the bone tissue (femur) of the rats in the control and resveratrol groups revealed normal histological structure (–) (Figures 2A and 2B). The rats in the NaF group were found to have thinning of the bone trabeculae (++) in the bone tissues, and an increase in the cellular density in the epiphyseal growth plate (+++) (Figure 2C). The rats in the NaF + resveratrol group were found to have a slight thinning in the bone trabeculae (+) in the bone tissues, and a slight decrease in the cellular intensity in the epiphyseal growth plate (+) (Figure 2D). [Scoring of histopathological finding: – (negative), + (slight), ++ (moderate), and +++ (severe)].



Figures 2A-2D. A: control group showing the femoral growth plate and the bone trabeculae with a normal histological appearance (–)), **B:** resveratrol group showing the femoral growth plate and the bone trabeculae with a normal histological appearance (–), **C:** NaF group showing thinning of the bone trabeculae (++) and a reduced cellular density in the femoral growth plate of femur (+++), and **D:** NaF +resveratrol group showing a slight thinning of the bone trabeculae (+) and a slightly reduced cellular density in the femoral growth plate of femur (+). H & E staining, bar=20 µm. Scoring of histopathological findings: – (negative), + (slight), ++ (moderate), and +++ (severe).

DISCUSSION

Many places in the world are covered with volcanic ash and these regions are rich in F with increased F levels in soil, water, and plants. A high intake of F may cause chronic fluoride toxicity or fluorosis. Moreover, increased F levels may result from industrial activities. For many years it has been known that endemic fluorosis may occur in regions with high F levels in the natural water resources, in the soil and in plants.[4,6,17,18,21] F is known to cross cell membranes and enter soft

tissues including skeletal and cardiac muscle, liver, skin, and erythrocytes.[7-12] Various changes occur in the blood, brain, kidney, liver, and spinal cord of animals after the chronic administration of F. These changes include abnormal behaviour patterns, altered neuronal and cerebrovascular integrity, metabolic lesions[8], thyroid dysfunction and hypothyroidism,[14-16] sinusoidal bradycardia,[17,18] stunted growth, hyperparathyroidism,[13] interference with mineral metabolism,[13,19] and low milk production in livestock.[1,12] Gao et al.[29] reported that the neurotoxic effects of long term F exposure include oxidative stress, changes in the cholinergic nervous system, and decreased ability in learning and memory. In animals, tenderness of the long bone epiphyses, pareses, demineralization, hypermineralization, bone fragility, and bone brittleness have been observed in fluorosis.[7,13] Apart from the relatively late anatomical lesions, F is responsible for metabolic disorders in various systems, organs, tissues, and individual cells.[1,4,11-14,18]

In the present study, as seen in Table 1, no anemia was observed in any of the groups. The hematocrit value and the hemoglobin level were increased in the resveratrol group compared to the control group. The platelet count and the vitamin A and D levels were significantly lower in the NaF group than in the control group. Compared to the control group, no statistically significant differences were observed in RBC, vitamin E, and vitamin K in the F-treated rats. Some studies have reported that F decreased the red blood cell[30-33] and platelet counts,[34] altered the neutrophil ratio,[35] and caused changes in blood parameters such as Hb, Hct, MCV, MCH, and MCHC.[30,36] In the present study, the increase in the hematocrit value and the hemoglobin level with resveratrol is normal and is due to the strong antioxidant effect of resveratrol.[20] The decrease in the platelet count and the vitamin A and D levels in the NaF group, compared to the control group, may be a consequence of NaF intoxication and chronic fluorosis. The positive effects of resveratrol can be seen clearly when the blood parameters and vitamin levels are examined in detail, and the hematological changes were ameliorated in the NaF + resveratrol group. In general, the changes in the blood parameters of the rats in the control and resveratrol groups are consistent with those reported in the literature.[37]

Inam et al.[38] reported an increase in liver enzymes in addition to mononuclear cell infiltration, ductus dilatation, and degenerative and necrotic hepatocytes in the portal areas in the liver histopathology with F administered in drinking water at a dose of 10 mg F/kg bw/day. In another study by Stawiarska-Pieta et al.,[39] perivascular mononuclear cell infiltration, sinusoidal and portal congestion, and necrosis in hepatocytes were detected in the livers of rats treated by the addition of F to their drinking water at a dose of 4 mg F/kg bw/day for 5 weeks. In the present study, although there were similar findings in the histopathological examination of the liver in the NaF group, necrotic hepatocytes were not found.

In a study by Chavassieux,[40] chronic exposure to NaF at toxic doses were shown to increase calcium uptake and retention in bones, and to stimulate osteoblasts, leading to erosion and morphological changes in bone tissue. In other studies,[41,42] exposure to toxic concentrations of F in drinking water for a long time caused skeletal lesions and negative changes in the bone structure of

humans. Cheng and Bader[43] reported that rats with experimentally-induced chronic fluorosis had increased bone mineral density in bone tissues and that their bones were more fragile. In a study conducted by Kierdorf,[44] yearling red deer stags from a fluoride-polluted region in North Bohemia (Czech Republic) were compared to control animals from two uncontaminated areas in West Germany. The deer from the F-contaminated area had elevated F levels in their antlers (845±257 mg F/kg ash) and pedicles (1448±461 mg F/kg ash) and osteosclerosis, osteomalacia, and osteoporosis were common. In our study, the finding of osteomalacia and osteoporosis-like changes is consistent with the studies mentioned above in which bone histopathology was examined and in which a significant decrease in osteoclast numbers and osteoclast density in the growth plate were found.

CONCLUSION

According to the changes in the blood parameters and the histopathological findings of the present study, it can be concluded that resveratrol may have a beneficial effect in ameliorating the liver and bone damage caused by chronic fluorosis.

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