Fruit Extract of Syzygium cumini Cures Toxic Effects of Fluoride on Erythrocytes and Femur Bone in Male Mice

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ABSTRACT

Rescuing potentials of jambul (Syzygium cumini; old name: Eugenia jambolana) fruit pulp extract (JFPE) on erythrocytes count and various morphometric parameters of femur bone against sodium fluoride (NaF) exposure were investigated. Forty Swiss Webster albino male mice (Mus musculus) were distributed equally in 4 groups: control; NaF; Jambul and NaF+jambul. Control and jambul groups were given F (fluoride) free water (15 days). F-ions (50ppm) from NaF in drinking water were given to NaF and NaF+jambul groups for 10 days and F free water for next 5 days. Animals in jambul and NaF-jambul groups also received 0.25mL JFPE (twice daily) on days 11-15. Anatomically considerable fluorosis of the femur on F exposure was noted. Moreover significantly (P<0.05) higher femur density (mg/mL) was noted in NaF (259.4±0.025) than control (174.53±0.01), jambul (199.48±0.013) and NaF-jambul (179.3±0.014) groups. The other parameters (i.e. Femur weight before and after de-mineralization, shaft length and diameters of femur shaft and head) showed significant (p<0.05) decrease in NaF than that of the control group. Moreover a significant (p<0.05) decline in erythrocyte count (EC) (Millions/mm³) in NaF (4.072±0.094) than control (5.358±0.08), jambul (5.428±0.084) and NaF-jambul (4.794±0.16) groups was noted. The findings show that post treatment with jambul pulp extract for 5 days have shown retrieval capacities upon the debilitating effects of F exposure in bone parameters and EC. Based upon these results it can be concluded that JFPE possesses superb curative potentials for toxic effects of F on EC and bone parameters of adult male mice.

Key words: Erythrocyte count, Femur, Fluoride, Syzygium cumini (jambul)

INTRODUCTION

Despite its potential benefits in bone development in terms of increased mineralization and simultaneously decreased resorption, F exposure has been held responsible for various toxicological manifestations (Grynpas & Cheng, 1988; Palmer & Wolfe, 2005). These include hematological damage, endochondral ossification causing mineralization of cartilage, osteomalacia and diminished bone strength in spite-of increased bone density (Segaard et al., 1995; Turner et al., 1996; Inoue et al., 2006; Agalakova & Gusev, 2011; Ahmad et al., 2012; Nabavi et al., 2013).

Jambul fruit has been found to contain high levels of anthocyanins, flavonoids, phenolics, carotenoids and vitamins that help in free radical scavenging activity thereby diminishing degenerative changes and macromolecular oxidation (Kubola et al., 2011; Chaudhary & Mukhopadhyay, 2012). Jambul fruit pulp has been found to significantly improve the hemoglobin contents and erythrocyte count as compared to the control animals (Lohar et al., 2009). Moreover, jambul extract has also been shown to cause significant reduction of abnormal sperm production (Tripathi et al., 2013). Additionally, some preclinical trials have also indicated the chemopreventive and radioprotective properties of jambul (Swami et al., 2012).

In present study, we are reporting the F exposure effects upon the osteo-hematological parameters in relation to the corrective potentials of JFPE.

MATERIALS AND METHODS

Chemicals

All chemicals used in the animal treatment (NaF), and laboratory preparations (ethanol, EDTA, glacial acetic acid, formaldehyde, wax and Canada balsam) and the stains (hematoxylin and eosin) used in this study were of the analytical (lab) grade.

Jambul (Syzygium cumini) Fruit-pulp Extract

Fully ripen jambul berries were used to get pulp extract. The washed dried fruits were thoroughly shaken to soften up the pulp and separation of seeds in wide mouthed lidded glass jar. Fresh pulp (100g) thus obtained was thoroughly mixed in 100 ml of drinking water for 5 min in chopper blender. The thick juicy mass obtained was centrifuged at 500 rpm for 10-15 minutes for...
separation of dark violet supernatant from the insoluble fibrous material. The supernatant was stored at \(-30^\circ\text{C}\) in sterilized 1.5mL eppendorf tubes for animal consumption during experiments.

**Animals**

Forty (3–4 months old) male (Swiss Webster) mice weighing 30±2g, were used in this study. The ambient housing conditions were maintained at 12 hrs dark-light cycles, 50% humidity and 23±2°C temperature. Standard laboratory mouse feed and Fluoride free water were provided ad libitum. The animals were distributed equally in four groups as follows:

**Control**: The animals in this group were maintained on F free water (Kush Aab – a certified product of University of Sargodha) for all 15 days of the study.

**NaF**: Animals in this group were given 50 ppm F- ions in drinking water for first 10 days and F free water for last 5 days.

**Jambul**: The animals in this group were fed on F free water for all 15 days however 0.25ml of JFPE was given to them by gavage after every 12-hr intervals, for the last 5 days.

**NaF-jambul**: These animals were provided 50ppm F- ions in drinking water for first 10 days followed by F-free water and JFPE in last 5 days as mentioned in jambul group above.

Animals from all groups were euthanized through cervical dislocation to obtain femur bones and ventricular blood for erythrocyte count

**Femur, Treatment and Measurements**

Femurs were obtained intact (without any scraping or damage) from euthanized animal after removing skin and muscles. The bones were placed in a solution containing 70% ethanol and 0.5% KOH for 45 days to remove any remaining soft tissues (fleshy, tendinous and ligamentous materials). These were then immersed in acetone for 10 days to remove marrowfats. For decalcification each bone was placed separately in 5% aqueous EDTA solution in a 25ml air tight glass vial until the morphology of bone was completely lost and what remained behind was an aggregation of soft fibrous material.

Both femurs from each animal were subjected to the measurements of volume, length and diameters (prior to decalcification) and dry weights (prior and after decalcification). The mean value of both femurs of each animal, for the above parameters, was considered as unit data value. Appropriate instruments like 0.001g precision digital balance (Sartorius-TE214S), Vernier calipers (1/20mm precision) and screw gauge were used for the measurement of femur weight, length and diameter respectively. Liquid displacement method was used for volumetric measurements for this purpose, an instrument consisting of 1/100ml graduated glass pipette designed in our lab was used.

**Total Erythrocyte Count**

For erythrocytes count, blood from each animal was obtained directly from the right ventricle just after cervical dislocation in 3ml heparin treated plastic syringe. Following standard laboratory procedure, the erythrocytes were counted from the digital photomicrographs of the Neubauer haemocytometer containing blood samples.

**Statistical Analysis**

All data obtained were subjected to obtain group mean values ± Standard Error of the Means. For various parameters the data obtained were also analyzed with standard statistical methods of "(one way) ANOVA" and the "Duncan’s multiple range test" to obtain the significance of variations among the groups using SPSS software.

**RESULTS**

**Erythrocyte Count**

Statistical analysis (ANOVA) had shown highly significant variation among the groups (p<0.001). While the post hoc analysis revealed significant (p≤0.05) variation between NaF and NaF+jambul groups moreover these two groups also differed significantly with control and jambul groups (Table 1).
Table I: Erythrocyte count and various morphometric parameters of the femur bone in control; NaF; Jambul and NaF+Jambul groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups: Means±SEM</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Erythrocyte Count (Million/mm$^3$)</td>
<td>5.358±0.08</td>
</tr>
<tr>
<td>Weight (mg) of Femur (before decalcification)</td>
<td>46.1±0.0017$^*$</td>
</tr>
<tr>
<td>weight (mg) of Femur (after decalcification)</td>
<td>11.39±0.0002$^*$</td>
</tr>
<tr>
<td>Femur density (mg/ml)</td>
<td>174.53±0.01</td>
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<tr>
<td>Total length of femur (mm)</td>
<td>14.86±0.127$^*$</td>
</tr>
<tr>
<td>Femur shaft diameter (mm)</td>
<td>2.78±0.111</td>
</tr>
<tr>
<td>Width of the femur head (mm)</td>
<td>1.44±0.0239</td>
</tr>
</tbody>
</table>

$^*$Any two groups not sharing a common symbol from this set differ significantly (p≤0.05) from each other

**Femur Anatomy**

Perfect anatomical features of the femur were observed in control group animals; that include length, width and whitish color (indicating good ossification). The size and position of the third trochanter were also ideal. Similar features were observed in jambul group mice. The yellow coloration seen in the lower half of the femurs in NaF and NaF+jambul groups indicate fluorosis. However, the highest degree of fluorosis was seen in NaF group animals (Fig. 1).

**Weight of Femur (before decalcification)**

Statistical analysis had shown significant variation among the groups (P<0.05). Post hoc analysis also revealed significant difference (p<0.05) of control and jambul groups to that of the NaF and NaF+jambul groups (Table I).

**Weight of Femur (after decalcification)**

Statistical analysis had shown significant variation among the groups (p<0.05). Post hoc analysis revealed significant (p<0.05) variation between NaF and jambul groups while NaF and jambul groups also showed significant variation respectively with control and NaF+jambul groups (Table I).

**Femur Density**

Overall significant variations were found in the data pertaining femur density (p<0.05). Also post hoc analysis revealed significant difference (p<0.05) of the NaF to that of the control jambul and NaF+jambul groups (Table I).

**Total Length of Femur**

Statistical analysis had shown no significant variation among the groups. On the other hand post hoc analysis indicated significant (p<0.05) difference of NaF to that of jambul and control groups while NaF+jambul did not differ significantly from any other group (Table I).

**Femur Shaft Diameter**

Overall significant variations were found in the data pertaining femur shaft diameter (p<0.05). Also post hoc analysis revealed significant difference (p<0.05) of the NaF to that of control, jambul and NaF+jambul groups (Table I).
Diameter of the Femur Head

Overall significant variations were found in the data pertaining diameter of head of the femur (p<0.05). Also post hoc analysis revealed significant difference (p< 0.05) of the NaF to that of control, jambul and NaF+jambul groups (Table I).

DISCUSSION

Fluoride has long been related with bone health and fluorosis (Palmer & Wolfe, 2005; Turner et al., 1996; Søgaard et al., 1995; Waddington & Langley, 1998; Mousny et al., 2008; Shim et al., 2011). The results obtained in this research work show significant alterations in various parameters of the femur bone. As reported earlier, our finding of significant increase in bone density clearly indicated a general rise in compactness of bone on F exposure (Inoue et al., 2006; Søgaard et al., 1995; Shim et al., 2011). On the other hand, the simultaneous decline in femur weight indicates an F exposure related net significant loss of hydroxyapatite. Moreover, the significant increase in the non-mineral dry mass component of femur on F exposure indicate a decrease in the organic architecture that mainly consists of glycosaminoglycan chains and cellular components (Waddington & Langley, 1998). It is well documented that F affects the structural integrity of bone through its gradual incorporation in the mineralized part transforming hydroxyapatite crystals into hydroxyfluorapatite (Palmer & Wolfe, 2005; Inoue et al., 2006). Furthermore, NaF exposure has been linked with the production of reactive oxygen species causing apoptosis in mouse embryonic stem cells (Nguyen et al., 2012). Thus, increase in ROS production on F absorption in femurs must have caused the loss of organic components. As necrosis is always linked with the release of hydrolytic enzymes, this consequential F related death of osteocytes seem to be responsible for the loss of mineralized bone component (Zhang et al., 2013). In the same context, significantly decreased lowered mean femur length and diameter recorded in NaF than control group provide supplementary indications to our understanding that F exposure has caused a general loss in femur bone components. The significant decrease in femur head diameter on F treatment may be a partial outcome of the rapid loss of head cartilage and its simultaneous gradual mineralization (Inoue et al., 2006).

Our finding of significant decline in erythrocytes count on F exposure seems to be justifiable based upon the recent claims where F has been found to induce death of rat erythrocytes in vitro (Agalakova & Gusev, 2011). Moreover, in a recent in vivo study, exposure to NaF (600ppm) in drinking water for 7 day was found to cause significant increase in lipid peroxidation and highly significant depletion of reduced glutathione with many folds depressed activity of antioxidant enzymes (superoxide dismutase and catalase) in erythrocytes (Nabavi et al., 2013). Thus, it is proposed that the increased simultaneous oxidative stress and lipid peroxidation must have caused apoptosis leading to a significant decline in erythrocyte count.

While erythrocyte count has shown significant improvement, all the injurious effects of F exposure were found reclaims except the mineralized weight of the femur on JFPE post treatment indicating its reclamation potential for osteological and hematological losses of F.

Conclusion

Our findings indicate that F exposure at 50ppm or more for 10 days can cause a significant decline in erythrocyte count in male mice. Furthermore, with a simultaneous increase in density, this exposure leads to a significant decrease in the organic matrix and mineralized components of the major weight bearing bone (the femur). However, these deleterious inflications of F exposure were convincingly cured on post treatment of JFPE- indicating its medicinal potential.

REFERENCES


