Effects of sub-acute fluoride exposure on discrete regions of rat brain associated with thyroid dysfunction: a comparative study

Chaitali Sarkar and Sudipta Pal*

Nutritional Biochemistry Laboratory, Department of Human Physiology, Tripura University, Suryamani Nagar, West Tripura 799022

*Correspondence Info:
Dr. Sudipta Pal
Assistant Professor,
Nutritional Biochemistry Laboratory,
Department of Human Physiology,
Tripura University, Suryamani Nagar, West Tripura 799022
E-mail: sudiptapal@tripurauniv.in

Abstract

Objectives: In this present in vivo study, it is intended to examine the effects of fluoride on metabolic functions of four discrete regions of rat brain associated with thyroidal insufficiency.

Background: Fluoride contamination in drinking water is a major health issue. Adverse health effects of fluoride include dental and muscle fluorosis, lowering mental proficiency, neurological disorders and oxidative stress in soft tissues including brain. Thyroid is mainly concerned with regulation of metabolic homeostasis and thus supposed to be altered by fluoride toxicity.

Rationale: Earlier observations indicate that fluoride toxicity imposes certain adverse effects on brain metabolic activities. Alteration in metabolic profile in brain may be affected by the thyroid gland as because this gland takes care of the overall metabolic integration of the body.

Significance: The present study explores the detail mechanism of metabolic alteration of different parts of rat brain namely cerebrum, cerebellum, pons and medulla by fluoride, and also evaluates through a comparative analysis that which region is mostly affected by it. Furthermore, this study also suggests a link between brain metabolic profile and thyroid function.

Methods: Male rats of Wistar strain (N=6) were orally fed with 20 mg/kg/day fluoride for 30 days.

Results: Following fluoride exposure, total protein content depleted more significantly in cerebrum and medulla as compared with other brain regions. The proteolytic enzyme activity was severely affected by fluoride, especially in medulla. Changes in acidic, basic and neutral protein contents reveal that fluoride altered those parameters in a tissue specific manner. Nucleic acid contents were markedly reduced in medulla and pons by fluoride, whereas RNase activity increased in the respective brain regions. Protein carbonylation is pronounced in cerebral tissue. The neurotransmitter level was markedly reduced in cerebellum in comparison with others. Additionally, fluoride also altered thyroid metabolism as specified by depletion of nucleic acid contents and inhibition of the activities of thyroidal enzymes along with significant decrease in serum T3 and T4.

Conclusion: It is suggested that fluoride altered metabolic homeostasis in cerebrum, cerebellum, pons, medulla in a tissue specific manner that might be correlated with thyroidal insufficiency.

Keywords: Sub-acute fluoride exposure, brain protein and nucleic acid contents, proteolytic enzyme activities, thyroid hormones, thyroidal metabolic enzymes.

1. Introduction

Fluoride is recognized as an important natural and industrial environmental pollutant [1]. Groundwater contains variable concentration of fluoride depending upon the nature of the rocks and the occurrence of fluoride-bearing minerals [2]. It is continually used in aluminium industries, in the manufacture of fluoridated dental preparations and in the fluoridation of drinking water, thus increasing the risk of fluoride exposure to human being [2]. Additionally, other daily sources of fluoride exposure are food, fluoride additives, toothpastes and professional administration of fluoride gel [3]. Both acute and chronic exposure of fluoride affect human health. Fluorosis is a common term of health complications that generally occurs in case of long-term exposure of fluoride. It is generally categorized into three types, viz., skeletal fluorosis (affecting bones), dental fluorosis (affecting teeth) and non-skeletal fluorosis affecting liver, kidney, lungs, blood cells, reproductive cells, gastrointestinal mucosa and...
nervous system (brain and spinal cord) etc. [4]. Among various adverse health effects of fluoride, abnormal behavioural pattern, impairment of neuronal and cerebrovascular integrity and metabolic lesions are common [5, 6], indicating that fluoride severely affects brain function. The underlying mechanism of cellular toxicity imposed by fluoride involves generation of free radicals that may disturb cellular functions via induction of oxidative stress [7]. Oxidative stress is detrimental to tissue as it rapidly degrades cellular components like tissue proteins, nucleic acids, membrane lipids and also perturbs endogenous antioxidants [8]. Oxidative damage of nuclear and mitochondrial DNA in human brain is supposedly involved in mild cognitive impairments that establish relevance of integrity of brain DNA to brain function [9]. It is further suggested from the earlier studies that fluoride mostly affects soft tissues including brain [10] due to easy permeability across the cell membrane [11] as well as due to presence of more unsaturated fatty acids, high oxygen utilization, high iron content and decreased activities of detoxifying enzymes in that tissue [12]. Other than these, change in neurotransmitter level may also be involved in altering brain metabolic functions [13].

Epidemiological studies indicate that children from certain villages of China with high fluoride contamination in food and drinking water were suffering from low IQ level [2]. It is supposed that metabolic stress in brain tissue especially alteration in protein and nucleic acid by fluoride may be one of the causative factors for such adverse health effects. This assumption was confirmed by the studies of Trivedi et al. [14], which revealed a significant dose-dependent reduction of acidic, basic, neutral and total protein contents in the cerebral hemisphere, cerebellum and medulla oblongata regions of mice brain following fluoride exposure. Each and every part of the brain is assigned to specific physiological function. Cerebrum is mainly concerned with locomotor activity, sensory processing, learning and memory, language and communication; whereas cerebellum contributes coordination in movement, fine movement, motor learning, posture, equilibrium maintenance. Medulla mainly deals with the involuntary functions of breathing, heart rate and blood pressure, and the functions of pons include sensory roles in hearing, equilibrium, taste, and in facial sensations such as touch and pain, as well as motor roles in eye movement, facial expressions, chewing, swallowing, and the secretion of saliva and tears. However, it is presumed that any severe change in the metabolic efficacy of those brain regions may be reflected in their functional status.

Other than brain, effects of fluoride on other organ systems are also documented. Among them thyroid gland appears to be sensitive to the deleterious effects of fluoride [15, 16]. The development of brain at neonatal as well as growing stages is largely affected by thyroid hormones [17, 18]. Moreover, thyroid hormones are mainly concerned with regulation of metabolic homeostasis of the body, and it is supposed that functional disturbances in thyroid gland may perturb this regulatory mechanism. As fluoride imposes certain adverse effects on brain metabolic functions, it may be possible to have some changes in thyroid gland in relation to maintaining general metabolic integration. Adverse effects of fluoride as observed earlier include structural and functional changes in thyroid follicular epithelial cells [19] characterized by a decline in the colloidal content, vacuolation and damage to the endoplasmic reticulum [20]. All these can disrupt the synthesis of thyroid hormones [21] that may influence brain development as well as its function.

The effects of any environmental toxicant on tissue depend on the dose and duration of its exposure as well as susceptibility of that tissue to that particular toxicant. In the present study a comparative analysis among four discrete brain regions is intended to be evaluated for better understanding the mechanism of fluoride toxicity on metabolic function in them by looking into certain important biochemical parameters of protein and nucleic acid metabolism along with regional neurotransmitter level. Furthermore, it is proposed to find out the correlation between brain metabolic changes with thyroidal function, if any following fluoride exposure at the present dose and duration. To address this lack of information, the objective of this study is to investigate the variations of fluoride mediated toxicity among cerebrum, cerebellum, pons and medulla of rat brain associated with thyroidal metabolic profile.

2. Materials and methods

2.1. Chemicals

Sodium fluoride (NaF, molecular weight 41.99) procured from Qualigen (India), other chemicals like, TCA, PCA, RNA, NADPH, Na₂, boric acid, sucrose, diethylether, GSH, DTNB, NADH, thiobarbituric acid, haemoglobin, EDTA, haematoxylin, leucine, BSA, H₂O₂, methanol, ethanol, DMSO, glutathione reductase, H₂SO₄, HCl, NaOH etc. were of analytical grade and purchased from Merck (India), SRL (India), Sigma–Aldrich (India). Ultrapure water by Millipore was used.
throughout the experiment to avoid metal contamination in preparation of reagents.

2.2. Animals and treatments

Experiments were carried out using male Wistar albino rats weighing 140–180 g that were fed a standard 18% protein (casein) diet with water ad libitum. Six animals were housed in each plastic cage under 12-h light/dark cycle (lights on at 08:00 am) at a constant temperature of 25 ± 2°C with 42 ± 5% relative humidity. The study protocol was in accordance with the guidelines for animal research and approved by the Ethical Committee of Tripura University. Twelve rats were randomly divided into two groups of six animals and treated as described below for 30 consecutive days. The control group received distilled water, the fluoride exposed group received drinking water with 20 mg/kg b.w./day orally for 30 days [22].

Group I – Control (received the vehicle only).
Group II – Fluoride-exposed (sodium fluoride at a dose of 20 mg/kg b.w./day orally for 30 days) [22].

After completion of treatment schedule, animals were sacrificed under light ether anesthesia. Whole brain and thyroid were removed, washed and perfused with normal saline to remove residual blood and then blotted dry. The cerebral hemisphere, cerebellum, pons and medulla regions of brain were dissected carefully, blotted free of blood, weighed to the nearest mg and utilized for study. Tissue was kept at -20 °C until biochemical analysis was performed.

2.3. Body weight and organo-somatic index (OSI)

Each animal was weighed periodically and the weights of whole brain and thyroid were recorded. From these values the OSI was calculated [23] by the following formula.

\[
\text{OSI} = \frac{\text{weight (g) of the organ}}{\text{Day 30 total body weight (g)}} \times 100
\]

2.4. Biochemical assays

2.4.1. Tissue protein content

The amount of acidic, basic, neutral and total protein was evaluated separately by the method of Shashi et al. [24] and Trivedi et al. [25]. Determination of these proteins was done spectrophotometrically by the method of Lowry et al. [26] using bovine serum albumin as standard.

2.4.2. Estimation of RNA and DNA from rat brain

The nucleic acid level in different regional tissues of rat brain (cerebrum, cerebellum, pons and medulla) and thyroid was determined by the method of Stroev and Makarova [27].

2.4.3. Assay of ribonucleolytic (RNase) activity

The RNase activity in specific brain regions was determined by the method of Josseffson and Lagerstedt [28] as modified by Ambellan and Hollander [29]. The enzyme activity was expressed in terms of µg of RNase/100 mg tissue.

2.4.4. Protein carbonyl content

Protein carbonyl content was measured according to the method of Levine et al. [30]. Results were expressed as nmol of DNPH-incorporated/mg protein based on the molar extinction co-efficient of 22,000/M/cm for alkaline aliphatic hydrazones.

2.4.5. Free amino acid nitrogen content

Free amino acid nitrogen present in the brain tissue was extracted by a method of Rosen [31] using leucine standard curve.

2.4.6. Pronase activity

Tissue pronase activity in rat brain (cerebrum, cerebellum, pons and medulla) was estimated by the method of Barman [32]. Tissue pronase activity was expressed in terms of µmoles of tyrosine per minute per mg tissue protein.

2.4.7. Trypsin activity

The trypsin activity was measured by the method of Green and Work [33], and the result was calculated from tyrosine standard curve.

2.4.8. Cathepsin activity

Cathepsin activity of rat brain (cerebrum, cerebellum, pons and medulla) was determined by the method of Pokrovsky et al. [34]. The enzyme activity was expressed in terms of tyrosine per minute per mg protein.

2.4.9. Alanine aminotransferase (GOT) and aspartate aminotransferase (GPT) activities

Tissue GOT and GPT activities were determined using a standard kit (Coral clinical systems, Goa, India) following the method of Reitman and Frankel [35]. Both the enzyme activities were expressed as units per mg of tissue protein.

2.4.10. Brain biogenic amine level

The levels of the catecholamines in different regions of brain tissue namely dopamine (DA), norepinephrine (NE) and serotonin were studied according to the procedure of Schlumpf et al. [36].

2.4.11. Estimation of serum T3 and T4 level

Serum thyroid hormones (T3 and T4) were assayed using chemiluminescence immunoassay (CLIA) kits. The sensitivity of T3 and T4 were expressed as ng/ml and µg/dl respectively.

2.4.12. Thyroid peroxidase (TPO) assay

Thyroid peroxidase activity in thyroid tissue was determined by the CLIA kits according to the method of Kaczur et al. [37]. The enzyme activity was expressed as ΔO.D/min/mg of sample protein.

2.4.13. Thyroidal Na⁺-K⁺-ATPase assay

Thyroidal Na⁺-K⁺-ATPase activity was assayed by the modified method of Chandra et al. [38]. The inorganic phosphate (Pi) liberated was determined by the method of Baginski et al. [39].
enzyme activity was expressed as nmoles of Pi liberated/min/mg protein calculated from a standard curve of potassium dihydrogen phosphate.

### 2.4.14.5'-deiodinase I (5'-DI) 5'-DI assay

Iodothyronine 5'-deiodinase type I (5'-DI) activity was measured according to the modified method of Chandra et al. [38]. The concentration of T3 in the ethanolic extract after 0 and 30 min of incubation was estimated by enzyme-linked immunosorbent assay (ELISA). The activity of 5'-DI was calculated as the difference of the 0 and 30 min O.D. values and expressed in terms of pmoles of T3 formed/mg protein.

### 2.4.15. Protein assay

Protein contents in the tissue homogenates and supernatant were extracted by the method of Lowry et al. [26].

#### 2.4.16. Statistical analysis

All the values are expressed as mean±S.D. (n = 6). Differences between two groups were tested and p value < 0.05 was considered significant [40].

### 3. Results

#### 3.1. Effect of fluoride on gain in body weight and OSI

The gain in body weight of rats during fluoride treatment was monitored to see whether subacute fluoride exposure had any significant effect on body weight gain. Results show that gain in body weight was significantly (p < 0.01) decreased (18.49%) by fluoride as compared with the control group (Table 1). Changes in organ weight in relation to body weight after fluoride treatment were represented by OSI (organo-somatic index) which indicates that the whole brain OSI was decreased by 20.37% (p < 0.01), whereas OSI of thyroid gland increased by 41.18% (p<0.001) after fluoride exposure.

#### Table 1: Effects of fluoride on body weight and organo-somatic index of rat brain and thyroid after fluoride exposure

<table>
<thead>
<tr>
<th>Studied regions</th>
<th>Control (6)</th>
<th>Treated (6)</th>
<th>% of change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>160.18±2.2</td>
<td>130.56±1.04</td>
<td>18.49 (a &amp; b)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Organosomatic index of brain (OSI)</td>
<td>1.08±0.024</td>
<td>0.86±0.026</td>
<td>20.37 (a &amp; b)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Organo-Somatic Index (OSI) of thyroid gland</td>
<td>0.017±0.004</td>
<td>0.024±0.008</td>
<td>41.18 (a &amp; b)</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

[Values are Mean ± S.D., p compared with control group, *** indicates p<0.001; Figure in the parentheses indicate the number of animals]

#### 3.2. Effect of fluoride on differential protein contents in cerebrum, cerebellum, pons and medulla

Determination of differential protein contents in discrete brain regions is important for evaluating protein metabolic efficacy in them. The acidic, basic, neutral and total protein contents were decreased in the cerebral hemisphere (78.03%, p<0.001) more adversely in comparison with medulla (72.66%, p<0.001), pons (71.49%, p<0.001) and cerebellum (71.24%, p<0.001) of rat brain (Table 2), whereas the basic protein level was diminished maximally in medulla by 84.27% (p < 0.001), as compared with pons (80.66%, p < 0.001), cerebellum (72.47%, p < 0.001) and cerebrum (67.89%, p < 0.001). The reduction of neutral protein contents were in cerebellum (71.19%, p<0.001) and medulla (72.60%, p<0.001), in comparison with the pons (67.5%, p<0.001) and cerebrum (57.83%, p<0.001). Finally, the total protein level was inhibited maximally by 74.46% (p < 0.001) in medulla and 74.33% (p<0.001) in cerebrum, followed by cerebellum (71.97%, p<0.001) and pons (72.24%, p<0.001) of rat brain.

#### Table 2: Effect of fluoride on protein contents in cerebrum, cerebellum, pons and medulla of rat brain

<table>
<thead>
<tr>
<th>Different protein contents</th>
<th>Studied regions</th>
<th>Control (6)</th>
<th>Treated (6)</th>
<th>% of change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic (mg%)</td>
<td>Cerebrum</td>
<td>11.29±0.85</td>
<td>2.48±0.58</td>
<td>70.03</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>11.51±0.62</td>
<td>3.31±0.46</td>
<td>71.24</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Pons</td>
<td>9.40±0.26</td>
<td>2.68±0.34</td>
<td>71.49</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>9.29±0.07</td>
<td>2.54±0.16</td>
<td>72.66</td>
<td>p***</td>
</tr>
<tr>
<td>Basic (mg%)</td>
<td>Cerebrum</td>
<td>4.36±0.74</td>
<td>1.40±0.22</td>
<td>67.89</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>3.85±0.28</td>
<td>1.06±0.25</td>
<td>72.47</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Pons</td>
<td>3.31±0.14</td>
<td>0.64±0.24</td>
<td>80.66</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>2.86±0.12</td>
<td>0.45±0.12</td>
<td>84.27</td>
<td>p***</td>
</tr>
<tr>
<td>Neutral (mg%)</td>
<td>Cerebrum</td>
<td>1.66±0.62</td>
<td>0.70±0.25</td>
<td>57.83</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>1.81±0.23</td>
<td>0.34±0.11</td>
<td>71.19</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Pons</td>
<td>1.60±0.3</td>
<td>0.52±0.12</td>
<td>67.5</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.46±0.12</td>
<td>0.40±0.11</td>
<td>72.60</td>
<td>p***</td>
</tr>
<tr>
<td>Total (mg%)</td>
<td>Cerebrum</td>
<td>17.18±0.92</td>
<td>4.41±0.45</td>
<td>74.33</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>16.59±0.68</td>
<td>4.65±0.34</td>
<td>71.97</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Pons</td>
<td>13.69±0.1</td>
<td>3.8±0.26</td>
<td>72.24</td>
<td>p***</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>13.55±0.26</td>
<td>3.46±0.14</td>
<td>74.46</td>
<td>p***</td>
</tr>
</tbody>
</table>

[Values are Mean ± S.D., p compared with control group, *** indicates p<0.001; Figure in the parentheses indicate the number of animals]
3.3. Effect of fluoride on tissue nucleic acid and amino acid nitrogen contents

Table 3 represents the changes in tissue DNA and RNA contents as well as free amino acid nitrogen concentration in different regions of rat brain (cerebrum, cerebellum, pons and medulla). The study reveals that DNA content decreased maximally by 36.84% (p<0.001) in pons as compared with the medulla (34.57%, p<0.001), cerebrum (29.58%, p<0.01) and cerebellum (28.26%, p<0.01). RNA content of fluoride-exposed animals was also decreased significantly in medulla (32.56%, p<0.001) followed by pons (33.33%, p<0.001), cerebrum (20.16%, p<0.01) and cerebellum (10.23%, p<0.05) respectively. The present study further reveals that the free amino acid nitrogen produced maximally in cerebellum (73.88%, p<0.001) followed by medulla (50.46%, p<0.001) and pons (42.27%, p<0.001) of fluoride-intoxicated rat brain.

Table 3: Effect of fluoride on free amino acid nitrogen, DNA and RNA contents in cerebrum, cerebellum, pons and medulla

<table>
<thead>
<tr>
<th>Studied regions</th>
<th>Control (6)</th>
<th>Treated (6)</th>
<th>% of change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free amino acid N content (µg of leucine /mg of tissue protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>200.92±3.14</td>
<td>349.36±2.72</td>
<td>73.88</td>
<td>p***</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>234.82±1.42</td>
<td>377.41±1.6</td>
<td>60.72</td>
<td>p***</td>
</tr>
<tr>
<td>Pons</td>
<td>247.29±3.44</td>
<td>351.81±1.92</td>
<td>42.27</td>
<td>p***</td>
</tr>
<tr>
<td>Medulla</td>
<td>315.36±3.48</td>
<td>474.48±3.9</td>
<td>50.46</td>
<td>p***</td>
</tr>
<tr>
<td>DNA (mg/100 mg of tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.071±0.005</td>
<td>0.05±0.006</td>
<td>29.58</td>
<td>p**</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.184±0.014</td>
<td>0.132±0.008</td>
<td>28.26</td>
<td>p**</td>
</tr>
<tr>
<td>Pons</td>
<td>0.076±0.004</td>
<td>0.048±0.004</td>
<td>36.84</td>
<td>p***</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.081±0.005</td>
<td>0.053±0.006</td>
<td>34.57</td>
<td>p***</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.172±0.004</td>
<td>0.131±0.005</td>
<td>23.84</td>
<td>p**</td>
</tr>
<tr>
<td>RNA (mg/100 mg of tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.176±0.008</td>
<td>0.099±0.007</td>
<td>20.16</td>
<td>p*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.158±0.006</td>
<td>0.109±0.004</td>
<td>41.05</td>
<td>p***</td>
</tr>
<tr>
<td>Pons</td>
<td>0.084±0.004</td>
<td>0.056±0.004</td>
<td>33.33</td>
<td>p***</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.086±0.004</td>
<td>0.058±0.010</td>
<td>32.56</td>
<td>p***</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.124±0.003</td>
<td>0.082±0.005</td>
<td>33.87</td>
<td>p***</td>
</tr>
</tbody>
</table>

[Values are Mean ± S.D., p compared with control group, *** indicates p<0.001; Figure in the parentheses indicate the number of animals] The changes in tissue DNA and RNA level of rat thyroid have been represented in Table 3. Results indicate that DNA content decreased by 23.84% (p<0.01) following exposure to fluoride. RNA content of fluoride-exposed animals was also decreased significantly in thyroid gland of rat. The decrease was found to be 33.87% (p<0.001) from control value.

3.4. Effect of fluoride on tissue proteolytic enzyme activities

Determination of proteolytic enzyme activities in brain tissue is important for finding the correlation with tissue protein content. The pronase activity was decreased significantly in all studied regions of rat brain (Fig. 1). The maximum decrease was found in medulla (59.26%, p<0.001). The pronase activity was found to be 50.32% (p<0.001) in pons, 25.26% (p<0.01) in cerebrum and 21.08% (p<0.01) in cerebellum. Fig. 2 represents that the cathepsin activity was decreased by 55.96% (p<0.001) up to the highest level in medulla and 41.05% (p<0.001), 33.54% (p<0.001) and 17.06% (p<0.01) in pons, cerebellum and cerebrum respectively after fluoride exposure. It is revealed from Fig. 3 that the medullar region of brain showing highest trypsin activity and cerebrum showing lowest trypsin activity of brain tissue by fluoride exposure.

Figure 1: Effect of fluoride on pronase activity in cerebrum, cerebellum, pons and medulla of rat brain

![Figure 1](image-url)

Values are Means±S.D., p compared with control group, *** indicates p<0.001, ** indicates p<0.01
3.5. Effect of fluoride on tissue transaminase enzyme activities

Estimation of brain transaminase activities is important marker of amino acid conversion and mobilization. Effects of fluoride on brain (Fig. 4 & 5) GPT and GOT activity reveal that GPT activity in the experimental group was increased. The maximum increase of GPT was found in cerebellum by 65.49% (p < 0.001). Cerebrum showed the lowest increase in the GPT activity (21.27%, p < 0.01). Medullar region and pons region of rat brain expressed the enzyme activity by about 42.72% (p < 0.001) and 23.08% (p < 0.01) respectively. Tissue GOT activities also increased up to the maximum level in cerebrum of fluoride-exposed animals by 67.93% (p < 0.001) and pons showed lowest activity by 36.44% (p < 0.001). 46.94% (p < 0.001) and 40.20% (p < 0.001) enzyme activity was found in cerebellum and medulla of rat brain respectively.
3.6. Change in tissue RNase activity and protein carbonyl content after fluoride exposure

RNase helps in degradation of ribonucleic acid and thus its activity measurement provides information regarding RNA content in brain tissue. The tissue RNase activity increased significantly in all studied regions of rat brain after fluoride exposure (Fig. 6). The maximum increase was found in cerebellum which was found to be 115.98% (P<0.001). The lowest activity was found in pons by about 107.69% (P<0.001) of fluoride exposed rat brain.

Values are Mean ± S.D., p compared with control group, *** indicates p<0.001

Figure 6: Effect of fluoride on tissue RNase activity in cerebrum, cerebellum, pons and medulla of fluoride-treated rat’s brain

Figure 7: Change in protein carbonyl content in cerebrum, cerebellum, pons and medulla of fluoride-treated rat brain

Values are Mean ± S.D., p compared with control group, *** indicates p<0.001
Carbonylation of protein is an indicative marker of oxidative protein damage. **Fig. 7** represents the result of the extent of protein carbonyl content in brain tissue (cerebrum, cerebellum, pons and medulla) of the control and experimental animals. Fluoride exposure significantly increased the protein carbonyl content by 133.58% in medulla (p<0.001). The lowest value of protein carbonyl content was found in cerebrum which was found to be 112.15% (p<0.001).

### 3.7. Change in brain biogenic amine level after fluoride exposure

Estimation of neurotransmitter level in brain tissue reflects its functional efficacy. Brain biochemical variables namely norepinephrine (NE), dopamine (DA), and serotonin (5-HT) in cerebrum, cerebellum, pons and medulla of the control and experimental animals. Furthermore, the 5’ deiodinase I enzyme activity was found to be decreased by 44.12% (p<0.001) as compared to the control group of the fluoride exposed rat brain. The inhibited activity of thyroid peroxidase (TPO) in thyroid tissue by 45% (p<0.001). Additionally, Na⁺-K⁺-ATPase activity in the fluoride-treated group decreased by 36.27% (p<0.001) as compared to the control group of animals. Furthermore, the 5’ deiodinase I enzyme activity was found to be decreased by 44.12% (p<0.001) from the control group after fluoride treatment.**Table 5**.

**Table 4**: Change in norepinephrine, dopamine, and serotonin contents of fluoride exposed rat brain in cerebrum, cerebellum, pons and medulla.

<table>
<thead>
<tr>
<th>Studied regions (ng/g of tissue)</th>
<th>Control (6)</th>
<th>Treated (6)</th>
<th>% of change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>416.4±2.68</td>
<td>356.62±1.21</td>
<td>14.36</td>
<td>p*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>644.12±2.91</td>
<td>406.28±1.20</td>
<td>36.92</td>
<td>p***</td>
</tr>
<tr>
<td>Pons</td>
<td>531.14±3.14</td>
<td>422.25±2.48</td>
<td>20.50</td>
<td>p**</td>
</tr>
<tr>
<td>Medulla</td>
<td>541.44±23.20</td>
<td>422±23.39</td>
<td>22.05</td>
<td>p**</td>
</tr>
<tr>
<td>Dopamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>890.12±2.04</td>
<td>659.87±1.84</td>
<td>25.87</td>
<td>p**</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>951.38±2.60</td>
<td>627.09±1.06</td>
<td>34.09</td>
<td>p***</td>
</tr>
<tr>
<td>Pons</td>
<td>934.52±2.42</td>
<td>657.26±1.70</td>
<td>29.67</td>
<td>p**</td>
</tr>
<tr>
<td>Medulla</td>
<td>926.34±4.23</td>
<td>642.06±3.56</td>
<td>30.69</td>
<td>p***</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>1846.28±3.91</td>
<td>1365.48±3.15</td>
<td>26.04</td>
<td>p**</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>1585.08±5.28</td>
<td>1106.26±4.58</td>
<td>30.21</td>
<td>p***</td>
</tr>
<tr>
<td>Pons</td>
<td>1525.56±2.89</td>
<td>1096.82±2.36</td>
<td>28.10</td>
<td>p**</td>
</tr>
<tr>
<td>Medulla</td>
<td>1517.15±3.44</td>
<td>1081.62±4.48</td>
<td>28.70</td>
<td>p**</td>
</tr>
</tbody>
</table>

[Values are Mean ± S.D., p compared with control group, *** indicates p<0.001; Figure in the parentheses indicate the number of animals]

### 3.8. Effect of fluoride on serum T3, T4 level and thyroidal Na⁺-K⁺-ATPase, 5’ deiodinase I and TPO activities

Determination of thyroid hormone level in serum and key metabolic enzyme activities in thyroid are important for evaluation of thyroid functions in response to fluoride. **Table 5** represents that sodium fluoride significantly decreased triiodothyronine (T3) level in serum by 57.89% (p<0.001). The thyroxine (T4) level was also reduced significantly in the serum samples of rats following exposure to fluoride (Table 5). The decrease was found to be 28.42% (P<0.01) compared to the control group.

**Table 5** further represents that sodium fluoride significantly inhibited the activity of thyroid peroxidase (TPO) in thyroid tissue by 45% (p<0.001). Additionally, Na⁺-K⁺-ATPase activity in the fluoride-treated group decreased by 36.27% (p<0.001) as compared to the control group of animals. Furthermore, the 5’ deiodinase I enzyme activity was found to be decreased by 44.12% (p<0.001) from the control group after fluoride treatment (**Table 5**).

**Table 5**: Effect of fluoride on thyroidal Na⁺-K⁺-ATPase, 5’ deiodinase I and TPO activities and serum T3, T4 level.

<table>
<thead>
<tr>
<th></th>
<th>Na⁺-K⁺-ATPase (nmol of Pi/min/mg of protein)</th>
<th>5’ deiodinase I (pmole T3/mg of protein)</th>
<th>TPO (AOD/min/mg of protein)</th>
<th>Serum T3 (ng/ml)</th>
<th>Serum T4 (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(6)</td>
<td>2.04±0.12</td>
<td>10.2±1.14</td>
<td>0.40±0.05</td>
<td>1.52±0.11</td>
<td>5.84±0.08</td>
</tr>
<tr>
<td>F-treated(6)</td>
<td>1.3±0.09</td>
<td>5.7±1.12</td>
<td>0.22±0.03</td>
<td>0.64±0.08</td>
<td>4.18±0.12</td>
</tr>
<tr>
<td>% of change</td>
<td>36.27</td>
<td>44.12</td>
<td>45</td>
<td>57.89</td>
<td>28.42</td>
</tr>
<tr>
<td>Significance</td>
<td>p***</td>
<td>p***</td>
<td>p**</td>
<td>p***</td>
<td>p**</td>
</tr>
</tbody>
</table>

[Values are Mean ± S.D., p compared with control group, *** indicates p<0.001; Figure in the parentheses indicate the number of animals]
4. Discussion

The present study was undertaken to investigate the sub-acute exposure of sodium fluoride on metabolic efficacy of brain tissue by looking into certain parameters of protein and nucleic acid metabolism, proteolytic and transaminase enzyme activities, biogenic amine level of discrete brain regions. Additionally, fluoride altered thyroid functions by changing hormone synthesizing machinery of thyroid gland and also suppressed the activities of key enzymes that are involved in iodine uptake and conversion of T3 to T4. As thyroid gland has great impact on overall metabolic efficacy of the body system, marked changes in its function were reflected in changing brain metabolic profile also.

Fluoride exposure at the present dose and duration significantly decreased body weight (17.36% decrease) as well as organo-somatic index (21% decrease) as compared to control rats (Table 1), indicating that change in organ weight in relation to body weight was significantly affected by sub-acute exposure to fluoride. Change in body weight might be due to low intake of food, breakdown of muscle and tissue proteins or degeneration of organ structure in fluoride-stressed animals [6, 22]. Decrease in weights of cerebral hemisphere, cerebellum and medulla oblongata might be due to decreased protein synthesis or increased proteolysis by fluoride [41, 22]. On the other hand, fluoride exposure at the same dose and duration significantly increased the organo-somatic index of rat thyroid gland, which may be due to enlargement of thyroid gland by fluoride either by hypertrophy or hyperplasia of thyroidal cells in experimental rats as a result of strong absorbing and accumulating capacity of thyroid gland for fluoride [19].

Alteration in protein synthesis by fluoride has been indicated by decreased contents of acidic, basic, neutral and total protein in cerebrum, cerebellum, pons and medulla as observed in our present study. Most of the proteins (basic, neutral and total) were decreased maximally in medulla, whereas acidic protein content was decreased prominently in cerebrum in comparison with other studied brain areas. Additionally, fluoride also shows similar reducing effect on total protein content in cerebrum as like medulla. It is suggested that medulla is highly sensitive to sub-acute fluoride exposure in respect with protein metabolizing efficacy in other brain tissue. This is indicated by the fact that protein carbonylation which is an important biomarker of oxidative denaturation of tissue proteins occurs most adversely in medulla. Oxidative stress induces modification of native amino acids side chains in proteins to carbonyl (aldehyde and ketone) derivatives leading to protein carbonylation, macro-molecular oxidation, resulting in free radical attack to membrane proteins and phospholipids causing membrane damage via induction of lipid peroxidation, mitochondrial membrane depolarization. As a result of oxidative breakdown of tissue proteins free amino acids were rapidly increased in that particular region of rat brain. Decreased protein content in medulla and also other brain areas is ascribed to either increased breakdown of proteins or decreased synthesis of it, which is considered as one of the important metabolic stress caused by fluoride. Moreover, inhibition of oxidative decarboxylation of branched chain amino acids is supposed to be involved in protein breakdown in fluorosis [42]. Additionally, decreased brain protein contents in fluoride exposed animals may result from decreased ability of brain tissue to synthesize amino acids as a result of suppressed activity of certain metabolic enzymes like glutamine synthetase and methionine activating enzymes [43]. Though free amino acid nitrogen level in brain tissue was elevated in all brain areas, it may not be properly incorporated in newly synthesizing proteins due to toxic insult, rather the amino acids may be quickly mobilized by the catalytic action of transaminases from the studied brain regions to other areas, causing substrate deficiency for synthesis of desired proteins in those specific brain regions. Consequently, activities of proteolytic enzymes like pronase, cathepsin and trypsin were suppressed after fluoride exposure. Changes in the proteolytic enzyme activity showed consistency with the changes in regional protein level indicating that proteolytic enzyme activity decreased more significantly in the medulla in comparison with other brain areas due to fluoride toxicity.

The present study further establishes that both DNA and RNA level in different brain regions are significantly decreased after fluoride exposure. The changes are more effective in medulla and pons as compared with cerebrum and cerebellum. Alteration in nucleic acid synthesis may be ascribed due to improper attachment of mRNA to the ribosome and supposed to be involved in fluoride-induced alteration of protein metabolism [44]. Disturbances in translational as well as transcriptional processes, mitotic cell division and chromosomal aberrations are evident in fluoride toxicity which may involve change in DNA/RNA, DNA/protein and RNA/protein ratios [45]. Additionally, fluoride causes DNA damage by oxidation, base alteration and strand breaks [46] that may perturb cellular function. Another suggestive mechanism for fluoride induced DNA damage may involve oxidative stress in various cell types [47, 48].
Fluoride induces mitochondrial dysfunction [49] promoting overproduction of ROS that is supposed to be involved in neurodegeneration [50]. Two possible mechanisms have been suggested regarding this i) direct attacks of the free amide group of DNA by fluoride and ii) indirect fluoride-induced free radical attack to the hydrogen bonds of DNA forming various DNA adducts [51]. On the other hand, decrease in RNA content may be attributed to either increased ribonucleolytic activity or decreased RNA synthesis in response to this toxicant. The present study further reveals that RNase activity increased significantly in all the studied brain areas. This may help in catalyzing the degradation of RNA into smaller components and thus influences the cellular content of RNA [52]. The RNase activity of different regions of brain appears to show a relation with the relative changes of the regional RNA contents. Suppressed protein synthesis in rabbit brain by fluoride was supposed to be involved in decreased RNA content in those tissues [53]. Similarly, decreased total protein content in medulla and pons region is in consistency with their regional RNA level. Thus, it may be suggested that accumulation of fluoride may affect significantly the regulation of RNA metabolism in medulla and pons, thus influencing their protein synthetic machinery that is reflected in terms of total protein content. However, in cerebrum and cerebellum RNA contents decreased more in comparison with the earlier mentioned brain regions, whereas their total protein contents decreased in a similar pattern as like pons and medulla. This indicates that change in protein level in cerebrum and cerebellum is not exclusively dependent on their RNA contents. Rather proteolytic enzymes may be responsible for changing the protein contents in those specific brain tissues.

Changes in biogenic amine levels show that NE, DA and 5HT decreased more adversely in cerebellum followed by medulla, pons and cerebrum upon fluoride exposure. This may be attributed to less availability of precursor amino acids in those brain regions to synthesize the neurotransmitters. The decreased biogenic amine level may either be due to decreased activity of enzymes involved in their synthesis like DOPA decarboxylase, dopamine β-hydroxylase and tyrosine hydroxylase or to the enhanced release of catechol-O-methyl transferase caused by increased neuronal activity [54]. It has been reported that the decrease in serotonin level may occur due to conversion of serotonin to melatonin to combat against fluoride-induced oxidative stress [55]. Depletion of biogenic amines upon fluoride exposure may hamper normal neurological activities of different brain region [51] including neurotransmission, cognitive and motor functions and other behavioral and physiological activities.

Other than brain, consumption of fluoride through drinking water may cause adverse effect on other soft tissue such as thyroid, causing tremendous distress on its function [56]. Brain development at neonatal as well as growing stages is largely regulated by thyroid hormones [57, 58], which also facilitate the maturation of granule neurons [59] and protect them from apoptosis [60]. Deficiency of thyroid hormones during the sensitive period of neurogenesis and neuronal migration can cause irremediable damage to various structures, leading to death of granule cells [61, 62], and blunted dendritic arborisation of Purkinje cells [63, 64]. The present study reveals that the administration of fluoride produces significant decline in the levels of serum T3, T4, thyroid DNA and RNA level and suppresses the activity of enzymes like TPO, Na+-K+-ATPase and 5’ deiodinase I, which is in consonance with several earlier findings [21, 62, 65, 66, 67]. The decline in the level of thyroid hormones could be due to deficient iodine uptake or interference in the synthesis of thyroid hormones [68, 69]. It was perceived that the structural resemblance between fluorine and iodine might enhance the uptake of fluorides, thereby affecting iodide uptake and ultimately affecting the synthesis of thyroid hormones [70]. Disturbed synthesis and secretion of thyroid hormones by fluoride and its interference in activity of enzymes that catalyze the conversion of thyroxine (T4) to active triiodothyronine (T3) as observed presently were also previously reported [62, 15]. According to our suggestion, the reasons for decreased level of T4 during fluoride intoxication might be due to inhibition of absorption of the iodine through the interaction of fluoride and/or insufficient synthesis and secretion of thyroglobulin and oxidized iodides from the thyroid gland owing to the structural changes of the thyroid follicle. Previous report also suggested that fluoride inhibits the activity of TPO in the thyroid gland [65]. The inhibition of TPO-catalyzed reaction as evident from present findings results in decrease in serum level of thyroid hormones. The suggested mechanism of action for enzyme inhibition may involve the conversion of thyroid peroxidase to a free radical that reacts with resorcinol moiety to produce a flavonoid radical [38] that could covalently bind to the catalytic amino acid residues on the enzyme, leading to enzyme inactivation [38]. In animal model, Boas et al. [71] reported that fluorinated compounds such as perfluorooctane sulphonate and perfluorooctanoic acid also inhibited TPO activity in rats, with reductions in T4 and T3. It is assumed that iodide
groups on TPO molecule may attract fluoride that causes decrease in the free active site on TPO molecule, leading to inactivation of this enzyme.

The decreased activity of Na⁺-K⁺-ATPase could adversely affect the accumulation of iodide in the thyroid, which is opposite to stimulation of Na⁺-K⁺-ATPase induced by hypothyroidism [72]. This effect might be due to accumulation of fluoride in the thyroid which directly inhibits the Na⁺-K⁺-ATPase [73] or the combined activity of fluoride and high TSH on activation of the protein kinase C, which decreases the activity of Na⁺-K⁺-ATPase [74]. Fluoride may cause internal injury to the cell membrane and affects the activity of membrane bound enzymes like Na⁺-K⁺-ATPase by disturbing membrane fluidity and membrane integrity and thus altering its permeability [75]. Both thyroidal and extra-thyroidal tissues like liver, kidney are the main site of generation of circulating T₃, which is produced by peripheral deiodination of T₄ to T₃. 5⁻-monodeiodinase I enzyme is responsible for this deiodination reaction. Sodium fluoride exposure at the present dose and duration reduces significantly the activity of 5⁻-monodeiodinase I, suggesting that fluoride decreases the rate of conversion of T₄ to T₃. All of these adverse effects of fluoride may be due to interference with synthesis or secretion of thyroid hormones by various mechanisms such as blockage of iodine uptake by the thyroid follicular cells, by inhibiting Na⁺-K⁺-ATPase activity, organification defect due to inhibition of thyroid peroxidase and suppression of thyroid hormone release.

Marked increase in DNA damage of thyroidal cells due to high fluoride and low iodine uptake has been reported [19]. In the present study, sub-acute exposure to fluoride caused damage to the cellular components like DNA, conforming earlier observation. DNA damage is supposed to be one of the reasons for high morbidity rates among those afflicted with hypothyroidism goiter and subcretinism in high fluoride and low iodine areas [76]. Additionally, RNA content in thyroid tissue significantly reduced after fluoride exposure. Fluoride-induced depression of RNA level in other tissue was reported earlier [44] which might be due to inhibition of nucleic acid synthesis and attachment of mRNA to ribosome after fluoride exposure. Moreover, fluoride, being an inhibitor of calcium and magnesium, the co-factors of certain metalloenzymes involved in nucleic acid biosynthesis reduces their synthesis [77]. Disturbed nucleic acid metabolism in thyroid tissue upon fluoride exposure is indicative of transcriptional and translational imbalance and chromosomal abnormalities as like brain tissue [78]. Alteration of nucleic acid metabolism in thyroidal cells might be involved in fluoride-induced functional disorders of thyroid. From these observations it is thus suggested that sub-acute fluoride exposure significantly affected metabolic homeostasis in different parts of brain as well as in thyroid tissue promoting their functional imbalance.

5. Conclusion

Sub-acute fluoride exposure through drinking water causes significant alteration of organ weight (brain and thyroid) in relation to body weight. Oxidative protein damage is supposed to be involved in alteration of tissue protein content after fluoride exposure that is indicated by elevated protein carbonyl content in those brain tissues. Cerebrum and medulla were more prone to fluoride toxicity in terms of total protein depletion as compared with cerebellum and pons. As a result of substrate unavailability the proteolytic enzyme activities significantly decreased in all observed brain areas especially in medulla. Differential responses were observed in case of acidic and basic protein contents in those four brain areas, indicating that fluoride imposes its toxicity in a tissue specific manner. Additionally, nucleic acid metabolism was adversely affected by fluoride as indicated by depletion of DNA and RNA contents. Nucleic acid depletion was more pronounced in medulla and pons which might be a causative factor for less activity of RNase in those tissues. DNA damage is also found in thyroidal tissue after fluoride treatment. Changes in biogenic amine level in studied brain regions after fluoride exposure may be correlated with functional disturbances of those specific areas. In this respect cerebellum was mostly affected by depletion of biogenic amine level due to fluoride toxicity. Moreover, fluoride affects the activity of certain metabolic enzymes transaminase in brain as well as thyroid peroxidase, Na⁺-K⁺-ATPase, iodothyronine 5⁻-deiodinase I in thyroid. Thyroid hormone (T3 and T4) synthesis was also significantly affected by fluoride. These indicate that alteration of brain metabolic activity is closely associated with alteration in thyroidal function by fluoride.

6. Ethical permission

All the animal experiments were done with proper permission and according to the guidelines of Institutional Ethics Committee of Tripura University.

Conflict of interest: The authors declare no conflict of interest for this study.
Acknowledgement

The present research work has been funded by the Department of Biotechnology (DBT), Govt. of India [Grant No.BT/04/NE/2009]. The authors sincerely thank the Co-coordinator, Dr. Debasish Maiti, State Biotech Hub, Tripura University for providing infrastructural facility and reagents required to carry out this research work.

References


[32] Barman T E. Enzyme Hand Book. 1974; Vols.1,2; Suppl. 1, New York: Springer-Verlag: pp. 1


