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Abstract:
Aim: This study evaluated the protective effects of γ-aminobutyric acid (GABA), a non-protein amino acid and anti-oxidant, against fluoride-induced hypothyroidism in mice.

Main methods: light microscope sample preparation technique and TEM sample preparation technique were used to assay thyroid microstructure and ultrastructure; enzyme immunoassay method was used to assay hormone and protein levels; immunohistochemical staining method was used to assay apoptosis of thyroid follicular epithelium cells.

Key findings: Subacute injection of sodium fluoride (NaF) decreased blood T4, T3 and thyroid hormone-binding globulin (TBG) levels to 33.98 μg/L, 3 2.8 ng/ml and 11.67 ng/ml, respectively. In addition, fluoride intoxication induced structural abnormalities in thyroid follicles. Our results showed that treatment of fluoride-exposed mice with GABA appreciably decreased metabolic toxicity induced by fluoride and restored the microstructural and ultrastructural organisation of the thyroid gland towards normalcy. Compared with the negative control group, GABA treatment groups showed significantly upregulated T4, T3 and TBG levels (42.34 μg/L, 6.54 ng/ml and 18.78 ng/ml, respectively; \( P < 0.05 \)), properly increased TSH level and apoptosis inhibition in thyroid follicular epithelial cells.

Significance: To the best of our knowledge, this is the first study to establish the therapeutic efficacy of GABA as a natural antioxidant in inducing thyroprotection against fluoride-induced toxicity.

Key words: GABA, Thyroid function, Ameliorative effect, Hormones, Hypothyroidism

1 Introduction
The thyroid gland, one of the most important endocrine glands, controls energy usage, protein synthesis and sensitivity of the body to other hormones by producing...
thyroid hormones (THs), such as triiodothyronine (T3) and thyroxine (T4).\textsuperscript{1,2,3,4} Hypothyroidism is a common thyroid dysfunction characterised by decreased production of THs. It is associated with symptoms, such as tiredness, cold intolerance and weight gain. It has been established that hypothyroidism causes impairments of many organs. AF Ajayi et al. investigated the effect of experimental hypothyroidism on hypothalamic-pituitary-ovarian axis and liver function. Their results showed that hypothyroidism led to alteration in reproductive organs cytoarchitecture and lysis of the hepatocytes.\textsuperscript{5,6} In children, hypothyroidism delays growth and intellectual development.\textsuperscript{7} Hypothyroidism affects 2 out of every 1,000 (0.2%) men and 20 out of every 1,000 (2%) women.\textsuperscript{8} Lifestyle-associated problems, such as increased stress, irregular sleep, pollution, internet radiation and ageing can induce hypothyroidism and are associated with its increased incidence in recent times.

Treatment of hypothyroidism aims to provide the body with THs that it is lacking. Synthetic thyroxine, which is identical to T4, is commonly used for treating patients with hypothyroidism. Most patients with hypothyroidism need thyroid hormone therapy throughout their lives.\textsuperscript{9} However, long-term thyroid hormone therapy is often associated with side effects, such as angina pectoris. Some people with normal levels of THs may develop symptoms similar to those of hypothyroidism.\textsuperscript{10} Several studies have investigated whether synthetic T4 therapy could benefit patients showing such symptoms despite normal thyroid functions. However, none of these studies have reported a difference between synthetic T4 therapy and a placebo (sugar pill) in improving the symptoms of hypothyroidism or the well-being of patients. However, the synthetic hormone replacement therapy has no effect on immune abnormalities\textsuperscript{11} and cannot reduce the high serum titres of anti-thyroid autoantibodies. Long-term hormone therapy inhibits the release of thyroid-stimulating hormone (TSH) by significantly increasing TH levels, which in turn prevents the recovery of thyroid function. These adverse effects of hormone therapy have driven researchers to seek for new compounds without side effects for treating hypothyroidism and protecting the thyroid system.

\textsuperscript{\gamma}-Aminobutyric acid (GABA) has various biological properties, such as anti-anxiety, anti-hypertensive, growth-promoting\textsuperscript{12} and anti-oxidant properties\textsuperscript{13,14}. Development and application of GABA has gained popularity in recent times. The anti-oxidant activity of GABA may be used for treating hypothyroidism caused by the action of free radicals on thyroid follicular epithelial cells. Different biological properties of GABA are effective against different symptoms of hypothyroidism, such as fatigue, weight gain, cold intolerance, depression, memory impairment and heart rate disorders. Therefore, GABA may have an outstanding potential to be used for treating hypothyroidism. To date, few studies have assessed the effects of GABA on the thyroid system. However, none of these studies have investigated the effect of GABA on the thyroid gland systematically and comprehensively.

Biogenic GABA is non-toxic and safe and does not cause pollution. The present study examined the protective effects of GABA obtained from scallop fermentation liquid on sodium fluoride (NaF)-induced hypothyroidism and its pathogenesis. To address this, the study investigated the role of GABA in the regulation of thyroid follicular epithelial cell injury and hormone levels in a hypothyroid mouse model.
2 Materials and Methods

2.1 Preparation of GABA

Scallops were purchased from Nanshan aquatic product market, Qingdao, China. A new *Enterococcus avium* strain 9184 with a high ability to produce GABA was isolated from a naturally fermented scallop solution. The scallop solution was mixed with 1% sodium glutamate and was used as a medium for culturing *E. avium* to produce GABA. Fermentation was performed for 3 days to obtain a solution rich in GABA (3.71 g/L of the solution). A 732-type cation-exchange resin was used to purify GABA from the fermented solution by using a conventional amino acid separation method. The purity of the end-product was 63%. This purified GABA was used for treating fluoride-induced hypothyroidism.

2.2 Materials

T4, T3, TSH and thyroid hormone-binding globulin (TBG) ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute. TUNEL Staining Kit was purchased from Roche Company. Thyroid tablets were purchased from Laiyang Biochemical Pharmaceutical Factory (Shandong province). NaF, GABA and other chemicals used in the study were of analytical grade and were purchased from Sigma Reagent Company.

2.3 Experimental Animals

Adult male Kunming mice (weight, 18–22 g) used in the study were obtained from institute of drug inspection of Qingdao. The animals were fed a standard protein diet (22% protein, Mazuri 5E10) and pure water. The animals were kept in animal houses maintained at standard temperature (22°C–25°C) and humidity (50%), with alternating 12-h light/dark cycle, and were acclimatised to laboratory conditions for 1 week. All the experimental procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Ethical Committee and the protocols were approved by the Committee on the Ethics of Animal Experiments of the Institute of Oceanology, Chinese Academy of Sciences, Shandong, China. All efforts were taken to minimise the suffering of the animals. The animals were anesthetised using ether before blood sampling. Laboratory animal quality certificate code is scxk20140001.

2.4 Experimental Design

The animals (N = 180) were maintained in the laboratory conditions for 7 days before the experiment. The animals were fed a diet containing 22% protein because it is considered as an adequate dietary protein level. The animals were divided into 2 groups of equal average body weight. The animals in one group (N = 20) served as controls and were given pure water. The animals in the other group (N = 160) were given an oral dose of 50 mg/kg body weight (bw)/day of NaF for 30 days and served as models of fluoride-induced hypothyroidism. Determination of NaF concentration: the oral median lethal dose (LD<sub>50</sub>) value of NaF was found to be 191.4 - 353.3 mg/kg body weight for mice. For sub chronic toxicity test, NaF dosage should be 1/20 - 1/5 of LD<sub>50</sub>. Determination of GABA concentrations: receiving GABA orally at a dose of 1-50 mg/kg bw/day was proved having health functions or endocrine regulation effects on organisms. Therefore, the concentrations of GABA in this experiment were
determined as 5, 25 and 50 mg/kg bw/day. To evaluate the anti hypothyroidism effects of GABA, the mice in the fluoride-exposed group were divided into the following 8 groups (N = 20): negative control group (NCG, mice receiving only pure water for 14 days), positive control group (PCG, mice receiving thyroid tablet orally at a dose of 50 mg/kg bw/day for 14 days), low concentration of pure GABA (G0.1, mice receiving pure GABA orally at a dose of 5 mg/kg bw/day for 14 days), medium concentration of pure GABA (G0.5, mice receiving pure GABA orally at a dose of 25 mg/kg bw/day for 14 days), high concentration of pure GABA (G1.0, mice receiving pure GABA orally at a dose of 50 mg/kg bw/day for 14 days), low concentration of laboratory-separated GABA (LSG0.1, mice receiving laboratory-separated GABA orally at a dose of 5 mg/kg bw/day for 14 days), medium concentration of laboratory-separated GABA (LSG0.5, mice receiving laboratory-separated GABA orally at a dose of 25 mg/kg bw/day for 14 days) and high concentration of laboratory-separated GABA (LSG1.0, mice receiving laboratory-separated GABA orally at a dose of 50 mg/kg bw/day for 14 days). In the groups receiving laboratory-separated GABA, only GABA concentration was calculated.

2.5 Animal Sacrifice and Blood Samples Collection
After GABA treatment, the mice were sacrificed and their blood were collected immediately. The samples were kept at −80°C until further analysis.

2.6 Estimation of Blood Levels of T4, T3, TSH and TBG
Just before sacrificing the animals, blood samples were collected from the hepatic vein of each animal under ether anaesthesia. Total blood levels of T3, T4, TSH and TBG were assayed using ELISA. The levels of T3, T4, TSH and TBG were expressed as ng/ml, μg/l, μIU/l and ng/ml, respectively.

2.7 Microstructure, Ultrastructure and Apoptosis of Thyroid Follicular Epithelial Cells
After the sacrifice, the thyroid glands of the mice were removed and fixed overnight in 10% neutral-buffered formalin and 2.5% neutral-buffered glutaraldehyde. The glands were then dehydrated using graded ethanol. The tissues fixed in 10% neutral-buffered formalin were embedded in paraffin and 5-μm sections were cut and mounted on slides. Some slides were stained with haematoxylin and eosin while some were stained with Tunel staining reagent. Tissue histopathology and cell apoptosis were analysed using a compound microscope. Tissues fixed in 2.5% neutral-buffered glutaraldehyde were examined under an electron microscope.

2.8 Statistical Analysis
Data are expressed as mean ± SD. Single group statistical analysis was performed using Student’s t-test. Single factor analysis of variance between multiple groups was performed using Duncan methods. A P value of <0.05 based on at least ≥3 independent experiments was considered statistically significant. Software SPSS 19.0. was used for data analysis.

3 Results
3.1 Changes in Blood Levels of T4, T3, TBG and TSH
NaF significantly decreased the levels of T4, T3 and TBG. In NCG, sodium fluoride exposure decreased T4 level by 37.6% and (P < 0.05), T3 level by 70.6% (P < 0.01) and TBG level by 35% compared with that in the control group (P < 0.05; Fig. 1).
Treatment of mice with high concentration of GABA countered the fluoride-induced alteration of T3 level by 17.7% (P > 0.05), indicating that GABA exerted a better protective effect than thyroid tablet against fluoride-induced decrease in blood T3 level by restoring 52.9% (P < 0.01) T3 expression compared with that in the NCG.

GABA also increased T4 and TBG levels. This effect increased with an increase in the concentration of GABA (Fig. 1). Administration of GABA appreciably countered fluoride-induced changes in blood T4 level compared with that in the NCG. Mice receiving the thyroid tablet also showed 23.24% (P < 0.01) restoration of fluoride-induced alteration in serum thyroxine level. Administration of GABA appreciably countered fluoride-induced change in blood TBG level compared with that in the NCG. GABA exerted better protective effects than the thyroid tablet against fluoride-induced decreased in blood TBG level by restoring 34.7% (P < 0.05) TBG expression compared with that in the NCG.

TSH levels in the NCG increased significantly by 89.5% (P < 0.01) and were normal (P > 0.05) in groups receiving high and medium concentrations of GABA (Fig. 1) compared with those in the control group.

3.2 Microstructure of Thyroid Follicular Epithelial Cells

Histological analysis showed that compared with the control group, in negative control group, the height of thyroid follicular epithelial cells was significantly increased (Table 1) and the thyroid follicular epithelial cells appeared enlarged and hyperplastic (Fig. 2-b). Treatment with 50 mg/kg bw/day of pure GABA or laboratory-separated GABA
markedly inhibited fluoride-induced damage in thyroid follicles and restored follicular size, colloidal material and epithelial tissue architecture to resemble those in the control group (Fig. 2-d, 2-e).

Table 1 Changes in the height of thyroid follicular epithelial cells after treatment with GABA and thyroid tablets

<table>
<thead>
<tr>
<th>Epithelial cell height (μm)</th>
<th>Control group</th>
<th>NCG</th>
<th>PCG</th>
<th>G0.1</th>
<th>G0.5</th>
<th>G1.0</th>
<th>LSG0.1</th>
<th>LSG0.5</th>
<th>LSG1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial cell height</td>
<td>3.11±0.36c</td>
<td>5.00±0.46a</td>
<td>3.70±0.49b</td>
<td>3.97±0.54b</td>
<td>3.14±0.48c</td>
<td>3.04±0.22c</td>
<td>4.30±0.46a</td>
<td>3.38±0.27bc</td>
<td>3.02±0.23c</td>
</tr>
</tbody>
</table>

* The result is reported as means ± SD. Single group statistical analysis was performed using Student's t-test. Single factor analysis of variance between multiple groups was performed using Duncan methods. According to Duncan analysis, the letter “a” represents the groups whose mean values are relatively larger. From “a” to “c”, the mean values decrease in turn. There is no significant difference between groups with the same letter. There is a significant difference between groups with different letters, P≤ 0.05.
Fig. 2 Histological changes after treatment with GABA and thyroid tablets. (a) Control group, (b) NCG, (c) PCG, (d) G1.0 and (e) LSG1.0

3.3 Ultrastructure of Thyroid Follicular Epithelial Cells

Compared with thyroid follicular epithelial cells of mice in the control group, the cells of mice in the NCG showed irregularly shaped nucleus (Fig. 3-b-C), disappearing mitochondrial crests (Fig. 3-b-B), reticular expansion (Fig. 3-b-B), reduced microvilli (Fig. 3-b-A), chromosome concentration (Fig. 3-b-C) and increased lysosome number. GABA treatment inhibited fluoride-induced damage of thyroid follicles and restored mitochondrial integrity (Fig. 3-d-A, 3-e-CD), chromatin state (Fig. 3-d-D, 3-e-A), reticulum (Fig. 3-d-C, 3-e-D), microvilli (Fig. 3-d-B, 3-e-B) to resemble those in cells of mice in the control group. According to Table 2 and Figure 3, GABA treatment exerted better protective effects than thyroid tablets (Fig. 3-c) against fluoride-induced damage of the ultrastructure of thyroid follicles.

Table 2 Changes in the ultrastructure of thyroid follicular epithelial cells after treatment with GABA and thyroid tablets

<table>
<thead>
<tr>
<th>Group</th>
<th>Mitochondria number</th>
<th>Mitochondria size (μm²)</th>
<th>Length of microvilli (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.67±2.33a</td>
<td>0.22±0.04c</td>
<td>1.29±0.27a</td>
</tr>
<tr>
<td>NCG</td>
<td>8.00±1.15c</td>
<td>0.96±0.10a</td>
<td>0.33±0.05b</td>
</tr>
<tr>
<td>PCG</td>
<td>16.33±2.03b</td>
<td>0.59±0.06b</td>
<td>0.29±0.03b</td>
</tr>
<tr>
<td>G1.0</td>
<td>20.67±2.33b</td>
<td>0.24±0.05c</td>
<td>1.34±0.25a</td>
</tr>
<tr>
<td>LSG1.0</td>
<td>18.33±2.60b</td>
<td>0.23±0.04c</td>
<td>1.85±0.19a</td>
</tr>
</tbody>
</table>

* The result is reported as means ± SD. Single group statistical analysis was performed using Student's t-test. Single factor analysis of variance between multiple groups was performed using Duncan methods. According to Duncan analysis, the letter “a” represents the groups whose mean values are relatively larger. From “a” to “c”, the mean values decrease in turn. There is no significant difference between groups with the same letter. There is a significant difference between groups with different letters, P≤ 0.05.
Fig. 3 Ultrastructural changes after treatment with GABA and thyroid tablets. (a) Control group, (b) NCG, (c) PCG, (d) G1.0 and (e) LSG1.0.

3.4 Apoptosis of Thyroid Follicular Epithelial Cells

Staining with Tunel reagent showed apoptotic chromatin condensation as a shiny light green spot. Ratios of apoptotic cells to the total follicular epithelial cells after treatment with GABA and thyroid tablets are shown in Table 3. Changes associated with fluoride-induced apoptosis in thyroid follicular epithelial cells are shown in Fig. 4-B. GABA supplementation inhibited fluoride-induced apoptosis in thyroid follicular epithelial cells and restored the changes to resemble those in the control group. GABA exerted a better inhibitory effect on the apoptosis of thyroid follicular epithelial cells than the thyroid tablets. Anti-apoptotic effects of pure GABA and laboratory-separated GABA were almost similar (Fig 4-D, 4-E; Table 3).

Table 3 Ratios of apoptotic cells to the total follicular epithelial cells after treatment with GABA and thyroid tablets

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>NCG</th>
<th>PCG</th>
<th>G1.0</th>
<th>LSG1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis rate</td>
<td>0.74%±0.13c</td>
<td>12.97%±1.78a</td>
<td>7.54%±1.39b</td>
<td>3.25%±0.97c</td>
<td>3.45%±0.55c</td>
</tr>
</tbody>
</table>

* The result is reported as means ± SD. Single factor analysis of variance between multiple groups was performed using Duncan methods. According to Duncan analysis, the letter “a” represents the groups whose mean values are relatively larger. From “a” to “c”, the mean values decrease in turn. There is no significant difference between groups with the same letter. There is a significant difference between groups with different letters, P≤0.05.

Fig. 4 Apoptosis of thyroid follicular epithelial cells. (A) Control group, (B) NCG, (C) PCG, (D) G1.0, (E) LSG1.0 and (↑) apoptosis spot

4 Discussion

The present study evaluated the protective effects of GABA on NaF-induced
alteration of thyroid metabolism and the associated structural and functional changes in thyroid follicular epithelial cells in male Kunming mice.

Previous studies involving various model systems have shown that oxidative stress and oxygen-derived free radicals play an important role in the pathogenesis of NaF-induced hypothyroidism. Oxidative stress associated with fluoride toxicity causes DNA damage in various cell types. Excessive fluoride intake affects the functional status of the hypothalamic–pituitary–thyroid system, thus adversely affecting the synthesis of cellular metabolites, such as DNA and RNA in thyroidal cells. The thyroid gland is highly susceptible to the effects of fluoride ions, which decrease the concentrations of T3 and T4 hormones, thus inducing hypothyroidism. Fluoride causes thyroidal dysfunction, as reported in a study by Yu, which showed that decreased serum level of T4 and increased level of TSH in populations in a fluorosis endemic area were independent of iodine intake. Biological compounds with anti-oxidant properties protect cells and tissues against toxic stress. Anti-oxidant treatment protects cells from lipid peroxidation caused by fluoride exposure. Our findings showed that subacute fluoride exposure significantly altered the histological architecture of the thyroid gland, as evidenced by increased epithelial thickness and layers, reduced follicular cavity area and colloidal content. GABA treatment of fluoride-intoxicated mice almost completely restored the microstructure of the thyroid tissue. GABA, a powerful anti-oxidant, protected thyroid cells from oxidative stress-mediated cell damage. GABA increased the synthesis of T3 and T4 and decreased the apoptosis of thyroid follicular epithelial cells. Our results also showed that GABA changed the ratio of T3/T4, which ensured a stable and high activity of T3.

The liver is an important organ for the synthesis of TBG. TBG binds to circulating THs. It is one of 3 proteins (along with transthyretin and albumin) responsible for carrying T4 and T3 in the bloodstream. Of these 3 proteins, TBG has the highest affinity for T4 and T3 and carries majority of T4 in the bloodstream. Genetically, TBG functions as a serpin. However, it does not have any inhibitory activity like other members of this protein class. NaF also exerts toxic effects on liver cells, leading to liver failure, which in turn alters TBG production. GABA counters the effect of fluoride on the liver and prevents the decrease in putrescine level. Maintenance of cellular polyamine levels is one of the important protective functions of GABA in liver cells. Putrescine prevents liver injury. GABA promotes the proliferation of liver cells. A healthy liver guarantees accurate TBG production. If TBG level is high, it binds to more THs, thus decreasing free hormone levels in the bloodstream and leading to the proper stimulation of TSH and production of more THs.

The regulatory effect of GABA on TSH can be reflected in two aspects. In contrast, under the effect of sodium fluoride, the function of the thyroid follicular epithelial cells was blocked, and therefore, the level of THs in the blood decreased significantly. THs can promote the synthesis of a protein, which inhibits the secretion of TSH. Once the levels of THs decrease, the amount of this protein was also decreased, thus causing the pituitary gland to secrete more TSH. Therefore, the TSH level in the negative control group was significantly higher than that in the positive control group. TSH can promote the proliferation and hyperplasia of thyroid epithelial cells, turning the flat follicular epithelial cell into a cubic or columnar one. Prolonged high concentrations of TSH cause further
damage to the thyroid structure and function. According to the results obtained from the experiment, GABA can effectively improve the TH levels in the blood, thus preventing excessive TSH secretion and reducing the damage in the thyroid tissue caused by TSH levels above the normal range. In contrast, GABA may also have a positive effect on TSH secretion, which may be achieved by upregulating sex hormones. GABA stimulates the adenohypophysis to secrete luteinising hormone (LH). LH along with follicle-stimulating hormone stimulates the secretion of oestrogen. Oestrogen enhances the response of the adenohypophysis to thyrotropin-releasing hormone, which in turn increases TH levels. Oestrogen also increases the production of binding proteins in the liver. LH stimulates the development of Leydig cells and promotes the secretion of testosterone. Previous studies have shown that androgens, especially testosterone, exerts a strengthening effect on Na\(\pm\)K\(\pm\)-ATPase activity. GABA affects TSH level, which in turn enhance the effectiveness of the thyroid system indirectly.

5 Conclusion

Our results showed that pure GABA and laboratory-separated GABA exerted identical protective effects against fluoride-induced hypothyroidism, which increased blood levels of T4, T3 and TBG; protected thyroid follicular epithelial cells from oxygen radical and high concentrations of TSH and inhibited apoptosis induced by NaF. However, pure GABA, which is produced using a chemical method, contains small amount of residual toxins; hence, it is not suitable for long-term use. In contrast, biogenic GABA produced in our laboratory is non-toxic and safe and does not cause pollution. The findings of the present study indicate that this GABA may have therapeutic potential in hypothyroidism.

Acknowledgements

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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