Effects of a Long-Term Administration of Aqueous Extract of *Prunella vulgaris* L. on Survival, Spontaneous Thyroid Carcinoma and Neoplastic C-cell Hyperplasia in Rats

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Research

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Abstract

Background: The continued global rise in thyroid carcinoma calls for alternative prevention and treatment strategies. *Prunella vulgaris* L. (PV) is a herbaceous plant with a medicinal property in the treatment of thyroid gland dysfunction, but its influence on thyroid carcinoma is unclear so far. This study was designed to investigate the effects of aqueous extract of PV on survival, spontaneous thyroid carcinoma and its preneoplastic lesion in rats.

Methods: A total of 552 Wistar rats (half female and half male) were randomly assigned into 4 groups and given one of the following diets for 24 months: chow diet (control), 2.5 (low), 8.25 (middle) and 25 (high) g/kg bw PV diets. After intervention, serum metabolic parameters including indicators of liver and renal function, glucose and lipid profiles were measured. Histological examination was conducted to confirm the types of thyroid carcinoma and its preneoplastic lesion.

Results: After intervention, serum aspartate transaminase of male rats in high PV group decreased significantly. No statistical differences among groups in terms of survival, body weight and other metabolic parameters were detected. In the control, low, middle and high PV groups, 14, 14, 15 and 8 rats developed thyroid carcinoma, respectively. Medullary thyroid carcinoma (MTC) emerged as the most common histological type in both sexes. Although PV failed to decrease risk of total thyroid carcinoma or each histological type, the incidence rates of neoplastic C-cell hyperplasia (CCH, a preneoplastic lesion of hereditary MTC) in PV groups were lower than that of control, and the lowest was observed in high PV group, manifesting as 5.25-time decrease in female rats and 5.5-time decrease in male rats.

Conclusion: Our results suggested for the first time that, a long-term administration of aqueous extract of PV decreased the incidence of neoplastic CCH without impairing survival and metabolic parameters.

Background

Thyroid carcinoma is the most common malignant tumor in the endocrine system. With the development of ultrasound scan technology, increased exposure to radiation and change in body mass index, incidence of thyroid carcinoma has been rapidly and consistently rising worldwide [1–4]. Data of Chinese tumor surveillance indicated that the number of patients diagnosed with thyroid carcinoma increased by 137% from 2010 to 2013. Although the overall prognosis of thyroid carcinoma especially well-differentiated thyroid carcinoma is favorable, adverse side effects caused by regular treatment cannot be ignored. For instance, radioactive iodine therapy is associated with increased risk of secondary primary malignancy [5], while thyroxine suppression may lead to arrhythmias and bone loss [6]. Additionally, quality of life in thyroid carcinoma survivors is always lower as compared with the general population [7]. Thus, a more effective and safer therapy is needed for thyroid carcinoma.

A multitude of plants and plant extracts have been used as adjuvant drugs for the treatment of carcinoma, due to their remarkably anti-tumor activity and low toxicity. *Prunella vulgaris* L. (PV), also known as self-heal, is a perennial herbaceous plant that is widely distributed in Asia and Europe. Previous
study demonstrated that PV was rich in flavonoids, sterols, organic acids, triterpenoids, polysaccharide and phenolic acids [8]. In traditional Chinese medicine, PV has been applied to treat thyroid gland dysfunction, goiter and neck lump for more than one thousand years, and today its clinical application extends to headache, dizziness, herpetic keratitis and certain cancers [8–11].

Unfortunately, literature regarding effect of PV on thyroid carcinoma is extremely limited, even though consumption of PV benefits thyroid function [12]. The only published paper showed that PV was able to induce apoptosis in papillary thyroid carcinoma (PTC) cells and follicular thyroid carcinoma (FTC) cells in vitro, indicating a potential inhibitory effect of PV on thyroid carcinoma [13], but this should be further confirmed by more in vivo researches. In the present study, Wistar rats were administered with different doses of aqueous extract of PV for 2 years to investigate impacts of PV on survival, metabolic parameters, spontaneous thyroid carcinoma and its associated preneoplastic lesion. Findings of this research will provide preliminary data for the use of PV as a remedy for the prevention and treatment of thyroid carcinoma.

**Materials And Methods**

**Preparation of aqueous extract of PV**

Dried spikes of PV (lot number Q17201) were purchased from Guangzhou Caizhilin Pharmaceutical Co. Ltd (Guangzhou, Guangdong, CHN), and a voucher specimen (voucher number 0065456) was deposited at Guangdong Provincial Center for Disease Control and Prevention (Guangzhou, Guangdong, CHN). The aqueous extract of PV spikes was prepared by Wanglaoji Pharmaceutical Co. Ltd. (Guangzhou, Guangdong, CHN). Briefly, 76.9 g of PV spikes were boiled twice in 1L of distilled water, 1.5 hours for each. The filtrate of decocted solution was concentrated under reduced pressure at 55 ~ 60°C, followed by ethanol (70%) precipitation for more than 24 hours. After ethanol distillation, the supernatant of extract was concentrated again and freeze-dried to produce a powder. One g of PV powder was equivalent to approximately 20 g of spikes. High Performance Liquid Chromatography (HPLC) (Santa Clara, CA, USA) was used to measure PV characteristic component rosmarinic acid (RA). The result showed that the content of RA in PV was 8.34 mg/g, which was in accordance with the requirement of Chinese pharmacopoeia (2015 edition).

**Dosage information**

Different doses of PV aqueous extract-derived powder were added into chow diet to prepare special PV diets. The final concentrations of PV in diets were 2.5, 8.25 and 25 g/kg body weight (g/kg bw), corresponding to 10, 33 and 100 times as much as maximal daily dose of PV in adult recommended by the Chinese pharmacopoeia [14]. Both rat chow diet and special PV diets were produced and purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, CHN).

**Animal and study design**
A total of 552 4-week-old Wistar rats, half female and half male, were provided by experimental animal center of Southern Medical University (Guangzhou, Guangdong, CHN) and housed individually in a standard environment (20 ~ 26°C, 40 ~ 70% humidity and 12-hour light/dark cycle). After acclimatization for 1 week, rats were randomly assigned into 4 groups (69 female + 69 male per group) according to body weight, and given free access to drinking water and one of the following diets for 24 months: chow diet (control), 2.5 g/kg bw PV diet (low PV), 8.25 g/kg bw PV diet (middle PV) and 25 g/kg bw PV diet (high PV). Body weight and feed intake were recorded. At month 12, 24 rats (12 female and 12 male) in each group were euthanized, and the rest was euthanized at month 24. Fasting abdominal aorta blood and thyroid tissue were collected. The experiment approach was reviewed and approved by animal experiment ethics committee of Guangdong Provincial Center for Disease Control and Prevention.

Measurements of metabolic parameters

Abdominal aorta blood samples were centrifuged at 200 g for 20 minutes at room temperature to acquire serum samples, which were stored at -80°C. Concentrations of serum alanine aminotransferase (ALT), aspartate transaminase (AST), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), glucose, triglyceride (TG) and total cholesterol (TC) were determined by automatic biochemical instrument (Hitachi 7600-010, JPN) using corresponding commercial kits (all from Nanjing Jiangcheng Bioengineering Institute, Nanjing, Jiangsu, CHN).

Histological examination

Thyroid tissue was fixed in 10% neutral buffered formalin at 4°C overnight and then embedded in paraffin. Section (4 ~ 6 µm) of tissue was stained with hematoxylin and eosin (H&E) for the histological examination. Histological types of tumor and pre-cancerous lesion were confirmed by two pathologists using light microscopy.

Statistical analysis

Data are presented as means ± SD and analyzed by SPSS 17.0 (Chicago, IL, USA) and SAS 9.1.3 (Cary, NC, USA). Kaplan-Meier survival analysis and log rank test were applied to compare survival of rats among groups. Body weight and feed intake were analyzed by repeated measurement analysis of variance. Differences in metabolic parameters among groups were identified using one-way ANOVA followed by Least Significant Difference (LSD)-test. Incidence rates of thyroid carcinoma as well as neoplastic CCH were compared by Fisher’s exact test or χ² test. Statistical significance was taken at P<0.05.

Results

Long-term consumption of PV did not impair survival of rats

No death was observed at month 12. After the intervention for 2 years, 66, 69, 71 and 71 rats were alive in the control, low PV, middle PV and high PV groups, respectively. Although there was no statistically
significant difference in survival among groups (Fig. 1), a trend toward increase could be found in male rats supplemented with middle and high doses of PV (52.6% in the control, 61.4% in middle and high PV groups).

**Effects of PV on feed intake and body weight**

Fluctuations of feed intakes of rats were detected in all groups over the intervening period (Fig. 2A-B). However, it did not lead to marked influence on body weight, because changes of body weights in all groups were extremely similar, especially for male rats (Fig. 2C-D).

**Effects of PV on metabolic parameters**

At the end of intervention, levels of metabolic parameters containing ALT, TP, ALB, BUN, CREA, glucose, TG and TC demonstrated no significant differences among groups (Table 1). AST of male rats in high PV group decreased remarkably as compared to the control ($P<0.05$), whereas similar result was not found in female rats, indicating that the potential protective effect of PV on liver function might be dependent on sex. Taken together, long-term administration of PV would not interfere with liver and renal function as well as glucose and lipid metabolism.
Table 1
Effects of PV on metabolic parameters at month 24

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low PV</th>
<th>Middle PV</th>
<th>High PV</th>
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<tbody>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>26.7 ± 6.1</td>
<td>26.7 ± 4.6</td>
<td>30.1 ± 11.5</td>
<td>28.7 ± 7.0</td>
</tr>
<tr>
<td>Male</td>
<td>38.0 ± 16.1</td>
<td>30.9 ± 4.7</td>
<td>34.1 ± 11.8</td>
<td>33.7 ± 11.4</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>133.7 ± 20.3</td>
<td>124.1 ± 16.7</td>
<td>128.1 ± 30.6</td>
<td>124.1 ± 25.9</td>
</tr>
<tr>
<td>Male</td>
<td>157.5 ± 28.6</td>
<td>145.4 ± 17.7</td>
<td>146.8 ± 32.4</td>
<td>137.6 ± 17.8a</td>
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<tr>
<td>TP (g/L)</td>
<td></td>
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<tr>
<td>Female</td>
<td>55.7 ± 3.3</td>
<td>56.4 ± 2.6</td>
<td>55.1 ± 3.0</td>
<td>55.5 ± 4.4</td>
</tr>
<tr>
<td>Male</td>
<td>56.0 ± 1.6</td>
<td>54.9 ± 2.3</td>
<td>54.5 ± 3.6</td>
<td>55.3 ± 2.5</td>
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<tr>
<td>ALB (g/L)</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Female</td>
<td>24.4 ± 2.5</td>
<td>24.9 ± 1.1</td>
<td>24.2 ± 1.3</td>
<td>23.7 ± 2.8</td>
</tr>
<tr>
<td>Male</td>
<td>22.3 ± 2.1</td>
<td>22.6 ± 1.5</td>
<td>22.3 ± 2.4</td>
<td>22.7 ± 2.3</td>
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<tr>
<td>BUN (mmol/L)</td>
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<tr>
<td>Female</td>
<td>6.4 ± 1.2</td>
<td>6.6 ± 0.7</td>
<td>6.4 ± 1.3</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>Male</td>
<td>6.9 ± 1.2</td>
<td>6.9 ± 1.1</td>
<td>7.2 ± 1.3</td>
<td>7.4 ± 1.4</td>
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<tr>
<td>CREA (µmol/L)</td>
<td></td>
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<tr>
<td>Female</td>
<td>53.5 ± 4.1</td>
<td>53.4 ± 3.1</td>
<td>52.7 ± 3.6</td>
<td>54.0 ± 5.5</td>
</tr>
<tr>
<td>Male</td>
<td>55.4 ± 3.8</td>
<td>53.7 ± 4.9</td>
<td>56.1 ± 4.4</td>
<td>54.6 ± 4.4</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
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</tr>
<tr>
<td>Female</td>
<td>3.6 ± 1.0</td>
<td>3.9 ± 0.7</td>
<td>3.9 ± 1.0</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>Male</td>
<td>3.8 ± 1.3</td>
<td>4.1 ± 0.8</td>
<td>3.7 ± 0.8</td>
<td>4.2 ± 0.7</td>
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<tr>
<td>TG (mmol/L)</td>
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<tr>
<td>Female</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Male</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.5</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td></td>
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<tr>
<td>Female</td>
<td>2.1 ± 0.6</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>2.0 ± 0.5</td>
</tr>
</tbody>
</table>
At the end of 24-month intervention, blood samples of rats fed chow diet (control), low, middle and high PV diets were collected and the serum metabolic parameters were determined. Data are presented as means ± SD. One-way ANOVA followed by LSD-test was used to detect statistical differences among groups. *P<0.05 versus control.

**Effect of PV on spontaneous thyroid carcinoma**

None of rats developed thyroid carcinoma at month 12. At month 24, the numbers of rats diagnosed with thyroid carcinoma were 14, 14, 15 and 8 in the control, low PV, middle PV and high PV groups, respectively. The histological types of thyroid carcinoma found herein were follicular variant of PTC (FVPTC, Fig. 3A), medullary thyroid carcinoma (MTC, Fig. 3B) and poorly differentiated thyroid carcinoma (PDTC, Fig. 3C), among which MTC was the most common one in both female and male rats, while poorly differentiated thyroid carcinoma was detected only in male rats (Table 2). No secondary carcinoma was detected in thyroid. Unfortunately, PV failed to decrease the incidence of either total thyroid carcinoma or each histological type.
Table 2
Effects of PV on thyroid carcinoma and neoplastic CCH at month 24

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low PV</th>
<th>Middle PV</th>
<th>High PV</th>
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</thead>
<tbody>
<tr>
<td><strong>Thyroid carcinoma</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>FVPTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1(2%)</td>
<td>1(2%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Male</td>
<td>1(2%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>PDTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Male</td>
<td>1(2%)</td>
<td>1(2%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>MTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5(11%)</td>
<td>7(13%)</td>
<td>9(16%)</td>
<td>4(7%)</td>
</tr>
<tr>
<td>Male</td>
<td>6(11%)</td>
<td>5(9%)</td>
<td>6(11%)</td>
<td>4(7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6(13%)</td>
<td>8(15%)</td>
<td>9(16%)</td>
<td>4(7%)</td>
</tr>
<tr>
<td>Male</td>
<td>8(15%)</td>
<td>6(11%)</td>
<td>6(11%)</td>
<td>4(7%)</td>
</tr>
<tr>
<td><strong>Neoplastic CCH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10(21%)</td>
<td>3(6%)^a</td>
<td>4(7%)^a</td>
<td>2(4%)^a</td>
</tr>
<tr>
<td>Male</td>
<td>12(22%)</td>
<td>7(13%)^a</td>
<td>7(13%)^a</td>
<td>2(4%)^a</td>
</tr>
</tbody>
</table>

Incidence rates of thyroid carcinoma and neoplastic CCH in rats fed chow diet (control), low, middle and high PV diets were analyzed by Fisher's exact test or $\chi^2$ test. Data are presented as cases (rate). ^a $P<0.05$ versus control.

**Long-term consumption of PV decreased the incidence of neoplastic C-cell hyperplasia (CCH)**

It should be noteworthy that the number of rat with neoplastic CCH (Fig. 3D), a preneoplastic lesion of hereditary MTC [15], was significantly different among groups ($P<0.05$, Table 2). Incidence rates of neoplastic CCH in all PV groups were lower than that of control, and the lowest was observed in high PV group, manifesting as 5.25-time decrease in female rats and 5.5-time decrease in male rats.

**Discussion**
PV consumption did not lead to remarkably changes in survival, body weight and majority of metabolic indicators (ATL, TP, ALB, BUN, CREA, glucose, TG and TC) in both female and male rats. Meanwhile, a significant decrease in AST was detected only in male rats fed high dose of PV, which fitted well with findings of Qu et al [16] suggesting that PV has the potential to protect liver function, and this effect may differ by sex. In disagreement with previous animal studies [17–19], the hypoglycemic, hypolipidemic and renal-protective functions of PV were not observed in the present study. The discrepancy was possibly due to different animal model because all the aforementioned benefits were found in diabetic rodents. It seems that rodents with metabolic abnormality are more sensitive to PV treatment. Considering PV did not induce any impairment in survival and metabolism after the 2-year intervention, its long-term administration was supposed to be safe.

Although PV is a medicinal plant with protective effects against thyroid dysfunction and several types of cancer [9, 11, 12, 20], there is no proven benefit of PV as a means to inhibit thyroid carcinoma. In the present study, a total of 3 histological types of thyroid carcinoma were found in rats, namely FVPTC, PDTC and MTC, among which MTC was the most frequent. Unfortunately, incidence rate of each carcinoma was not reduced after PV intervention. Based on these results, however, it was difficult to confirm that PV was completely ineffective in preventing and treating thyroid carcinoma in human, because PTC and FTC, which account for more than 90% of total thyroid carcinoma in human [3], did not occur spontaneously in rats fed chow diet. Furthermore, PV was shown to be capable of inducing apoptotic cell death in both PTC and FTC cell lines by regulating B-cell lymphoma-2/Bcl-2-associated X protein/caspase-3 signaling pathway [13]. Thus effects of PV on PTC and FTC are yet to be examined using specific animal models, and only after that can we conclude whether PV is helpful to fight against thyroid carcinoma.

Of particular interest in our study was the observation that incidence of neoplastic CCH in PV groups especially high PV group was obviously lower as compared with the control. CCH is classified into 2 types: physiologic CCH and neoplastic CCH. As for neoplastic CCH, even though there is no consensus on its role in the development of MTC so far [21, 22], most researcher inclined to recognize neoplastic CCH as the precursor of hereditary MTC (e.g. family MTC and type 2 multiple endocrine neoplasia) [15] and sporadic MTC in some cases [23]. As a result, PV was supposed to be capable of reducing risk of hereditary MTC, but we noted that incidence rates of MTC among all groups were lack of statistical difference after PV intervention. This was possibly because MTC observed in the present study was closer to sporadic type rather than hereditary type. More researches especially human researches pertaining to the potential protective effect of PV on hereditary MTC are warranted.

The components in PV extract that were responsible for the inhibition of neoplastic CCH were unclear. Abundant studies have yielded a robust evidence for a causal role of RET proto-oncogene mutation in the development of neoplastic CCH and the subsequent hereditary MTC [24–26]. Activating transcription factor 4 (ATF4) is shown to suppress MTC by targeting RET gene in vivo and in vitro [27]. It happened that RA, the characteristic component of PV, was previously reported to activate ATF4 when potentiating
the therapeutic effect of MG132 on hepatocellular carcinoma [28]. Therefore, RA was likely to be a functional component in PV extract for suppressing neoplastic CCH, but it needs to be further confirmed.

**Conclusion**

In summary, the current study firstly showed that a long-term administration of aqueous extract of PV decreased the incidence of neoplastic CCH without impairing survival and metabolic parameters in both female and male rats. PV is a promising drug resource with the potential to reduce risk of hereditary MTC.

**Abbreviations**

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate transaminase; ATF4, activating transcription factor 4; BUN, blood urea nitrogen; CCH, C-cell hyperplasia; CREA, creatinine; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography; LSD, Least Significant Difference; MTC, Medullary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; PV, *Prunella vulgaris* L.; RA, rosmarinic acid; TC, total cholesterol; TG, triglyceride; TP, total protein.

**Declarations**

**Acknowledgements**

Not applicable.

**Authors’ Contributions**

JS, MZ and XY designed the study; JS, MZ, ZZ, BC, JT, JH, RZ and XH performed the experiments; BC, JT and RZ analyzed the data; JH, SEO and XY interpreted the results of statistical analysis; JS, MZ and ZZ drafted the manuscript; XY, XH and SEO revised the manuscript. All the authors approved to summit the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.
Ethics approval and consent to participate

The study on animal was approved by animal experiment ethics committee of Guangdong Provincial Center for Disease Control and Prevention.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

References


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**Figures**

**Figure 1**

Effects of PV on survival in female (A) and male (B) rats. Rats were fed chow diet (control), low, middle and high PV diets for 24 months. Survival of rats was analyzed by Kaplan-Meier survival analysis and log rank test.
Figure 2

Daily feed intake and body weight of rats during feeding period. Daily feed intake and body weight of female (A, C) and male (B, D) rats fed chow diet (control), low, middle and high PV diets were analyzed by repeated measurement analysis of variance. Data are presented as means ± SD.*P<0.05 versus control, **P<0.01 versus control.
Figure 3

Sections of thyroid tissues stained with H&E (100×). After treatments, thyroid tissues of rats in the control, low, middle and high PV groups were collected for the histological examination. (A) FVPTC; (B) MTC; (C) PDTC; (D) Neoplastic CCH.