Exp. Anim. 66(4), 345–356, 2017

—Original—

Evaluation of a TPTX model induced by ischemia

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Abstract: Parathyroidectomy (PTX), especially total parathyroidectomy (TPTX), is often recommended for severe secondary hyperparathyroidism (SHPT) if other medical treatments fail. Accurate identification and resection of parathyroid gland (PTG) tissue is the cornerstone of PTX. The establishment of a rat TPTX model would be beneficial for several applications but faces the same problems. In this experiment, we studied the mechanisms of ischemia for the accurate identification and excision of PTG tissue to establish TPTX rat models and to analyze the effects of surgical removal of PTG tissue as well as the effects of different types of water intake in rats on clinical indices. We found that the ischemia method had advantages when establishing a rat TPTX model. Removal of the PTG tissue resulted in significantly changed postoperative indices, and varying the types of water intake induced significant differences in these indices after removal of the PTG tissue. The absolute value of the difference between the serum calcium and phosphorus concentrations (|Ca−P|) accurately reflected the effect of removal of the PTG tissue and was superior to the calcium-phosphorus product (Ca × P); Ca × P accurately reflected the effect of varying the types of water intake in rats and was superior to the |Ca−P|.
Key words: Ca × P, |Ca−P|, ischemia, SHPT, TPTX

Introduction

Chronic kidney disease-mineral and bone disorder (CKD-MBD) is characterized by diseases associated with metabolic abnormalities of calcium, phosphorus, and parathyroid hormone (PTH), and secondary hyperparathyroidism (SHPT) is an important component of CKD-MBD [14]. SHPT patients with hyperplasia of the parathyroid gland (PTG) and increased PTH secretion have primary clinical manifestations, including skeletal system lesions and cardiovascular diseases [9]. According to the guidelines [14, 15], the current clinical treatments for mild to moderate SHPT include dietary phosphate restriction and pharmacologic treatment with phosphate binders and active vitamin D, among others, and for severe SHPT, if medical treatments fail, parathyroidectomy (PTX) has been recommended [12, 20].

There are three types of PTX: (1) subtotal parathyroidectomy (SPTX), (2) total parathyroidectomy (TPTX), and (3) total parathyroidectomy and autotransplantation (TPTX+AT). In severe SHPT, pathological changes of the parathyroid tissues include nodular hyperplasia or

(Received 18 March 2017 / Accepted 1 June 2017 / Published online in J-STAGE 3 July 2017)
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adenomatous changes, and low serum calcium and high serum phosphorus concentrations in patients are likely to promote the hyperplasia of surviving PTGs, increasing the risk of recurrence of SHPT and the difficulties associated with reoperation [21]. SPTX and TPTX+AT are both associated with high risks of recurrence [4, 21], so many doctors advocate TPTX, which has shown great advantages over both SPTX and TPTX+AT for not only better improvements of biochemical indices and clinical symptoms but also fewer complications and lower recurrence rates [4, 16–18, 21].

TPTX has good application value, as would a successful rat TPTX model. Rat TPTX models can provide animal experimental support for PTX in SHPT, aid in the establishment of rat models of hypoparathyroidism that provide carriers for transplantation of parathyroid tissues, and be used as animal models for studies related to conditions of altered calcium and phosphorus metabolism, such as hypoparathyroidism, CKD-MBD, or dental growth [2, 7]. Due to the anatomical characteristics of the PTG [10], the accurate intraoperative identification and resection of the PTG are key for clinically successful PTX and are a major focus of academic research, as the visual method usually used in surgery to identify the PTG has some limitations [1]. The rat TPTX model also faces these problems, and the identification and direct removal of suspected parathyroid tissues by the visual method would limit the successful establishment of models and lead to complications and affects on indices analysis. The existing literature on the establishment of a rat TPTX model has no accurate identification methods or descriptions of anatomical characteristics of the PTG and lacks detailed procedures and detailed analyses of the postoperative changes in indices [2, 7]. In this study, using the ischemia method through the ligation of suspected sites to accurately identify and excise the PTGs of rats, we attempted to observe the anatomical characteristics of the parathyroid tissues, establish rat TPTX models, improve the success rate of establishing models, and improve the long-term survival rate of rats after surgery. We also attempted to analyze the effects of surgical removal of the PTGs and varying types of water intake in rats on the postoperative indices. Considering the instabilities and changing characteristics of the serum calcium and phosphorus concentrations, we created a new index, |Ca−P|, which is the absolute value of the difference between the serum calcium and phosphorus concentrations and is different from the calcium-phosphorus product (Ca × P), and we tested the sensitivities of Ca × P and |Ca−P|. We tried to provide references for the establishment of animal models and indices analyses concerning the identification of PTG and clinically related diseases and surgery operations.

Materials and Methods

Animals and groups

Fifty-six 9-week-old male Sprague-Dawley (SD) rats (JOINN Laboratories, Inc., Suzhou, China) weighing 190–210 g were used in the present study. The rats were acclimated for one week before the experiments. All rats were fed the same solid forage to ensure their long-term survival. To analyze the effects of different contents of calcium and phosphorus intake, rats were provided with varying types of water with different contents of calcium and phosphorus including sterilized tap water and sterilized distilled water. The forage and water were added to ensure that each rat consumed about 45 g/day of forage and 40 ml/day of water. All rats were allowed free access to the water and forage.

Animal care followed the Guide for the Care and Use of Laboratory Animals and the Animal Experiment Guidelines of Fudan University. This study was approved by the Committee for Ethics of the Fudan University Experimental Animal Science Department (No. 20160865A006).

The 56 rats were randomly divided into the following 5 groups: group A, which included 10 rats without TPTX that drank tap water; group B, which included 10 rats without TPTX that drank distilled water; group C, which included 12 rats with TPTX that drank tap water; group D, which included 12 rats with TPTX that drank distilled water; and group E, which included 12 rats with TPTX that drank tap water in the preoperative period and distilled water in the postoperative period.

There were 12 rats that underwent TPTX in each of groups C, D, and E, and each group had 2 rats that served as backups. All rats were fed with the same ordinary rat solid forage (Jiangsu Xietong Biotechnology Co., Ltd., Nanjing, China), the calcium content, phosphorus content, and calcium to phosphorus ratio of which were 10–18 g/kg, 6–12 g/kg, and 1.2:1 to 1.7:1, respectively. The calcium and phosphorus contents in sterilized tap water were 0.80 mmol/l and 0.01 mmol/l, respectively. The sterilized distilled water did not contain calcium or phosphorus.

Process of TPTX

Anesthesia and exposure of the surgical field of view: The rats were anesthetized with the 0.5% pentobarbital sodium solution (Merck KGaA, Darmstadt, Germany) at a dose of 40 mg/kg via intraperitoneal injection. After anesthetization of the rats, making a skin incision along the midline of the neck, and moving the subcutaneous submandibular glands, sternohyoideus, and sternothyreoideus to the sides with microsurgical tweezers layer by layer under a stereomicroscope (CSOIF, Shanghai, China), the dark-red thyroid tissues were exposed and located on both sides of the thyroid cartilage and the tracheal rings.

Identification and resection of parathyroid tissues: The parathyroid tissues were preliminarily searched for by the visual method under the stereomicroscope in the middle or superior poles of lobes of the thyroid from the ventral side to the dorsal side. If the tissues could be found preliminarily, they were then verified by the ischemia method; if not, the ischemia method was used to find them at the suspected site. The lobe of the thyroid was clamped gently with microsurgical tweezers, and needles were inserted with suture thread in the upper and lower 1 millimeter of the suspected tissues or sides from the lateral side of the lobe to the middle. Then the two sutures were both knotted, blocking the blood supply between the two sutures, and the parathyroid tissues emerged and were identified based on their color turning white; the surrounding thyroid tissues had a dark red color. The surgical operation range was between the two ligations. The two sutures were subsequently lifted, and the entire parathyroid tissue was resected with curved microsurgical scissors. In this procedure, any remaining white parathyroid remnants should also be subjected to resection, and as much of the thyroid tissue as possible should be retained. The removed tissues were immediately fixed in 4% paraformaldehyde for histopathological evaluation. During the operation, we resected the parathyroid tissue of rats in groups C, D, and E after identification of the PTGs.

Postoperative treatments: After suture of the neck muscles and skin and resuscitation from anesthesia in a warm room, the rats were returned to the animal room to continue feeding.

Histopathological evaluations: The parathyroid tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and the tissue blocks were sectioned at 4 μm thickness. The paraffin sections were dewaxed and stained with hematoxylin-eosin stain (H&E) (Beyotime, Jiangsu, China), and for immunohistochemical staining, the sections were incubated with rabbit anti-rat calcium-sensing receptor (CASR) polyclonal antibody (Boster, Wuhan, China; 1:200 dilution).

Laboratory evaluations

Blood was taken from the eye angular vein of each rat in the 5 groups in the preoperative period and on postoperative days 1, 3, 7, 14, 21, 28, 35, 42, and 49; the blood samples were analyzed for the concentrations of serum calcium and phosphorus (Modular P800 automatic biochemical analyzer, Roche, Basel, Switzerland), and 10 serum samples from rats in which the PTGs were recognized and long-term survival was observed in each group were analyzed to determine their serum whole PTH concentrations (rat PTH ELISA detection kit, Qian Bi Biotechnology Co., Ltd., Jiangsu, China).

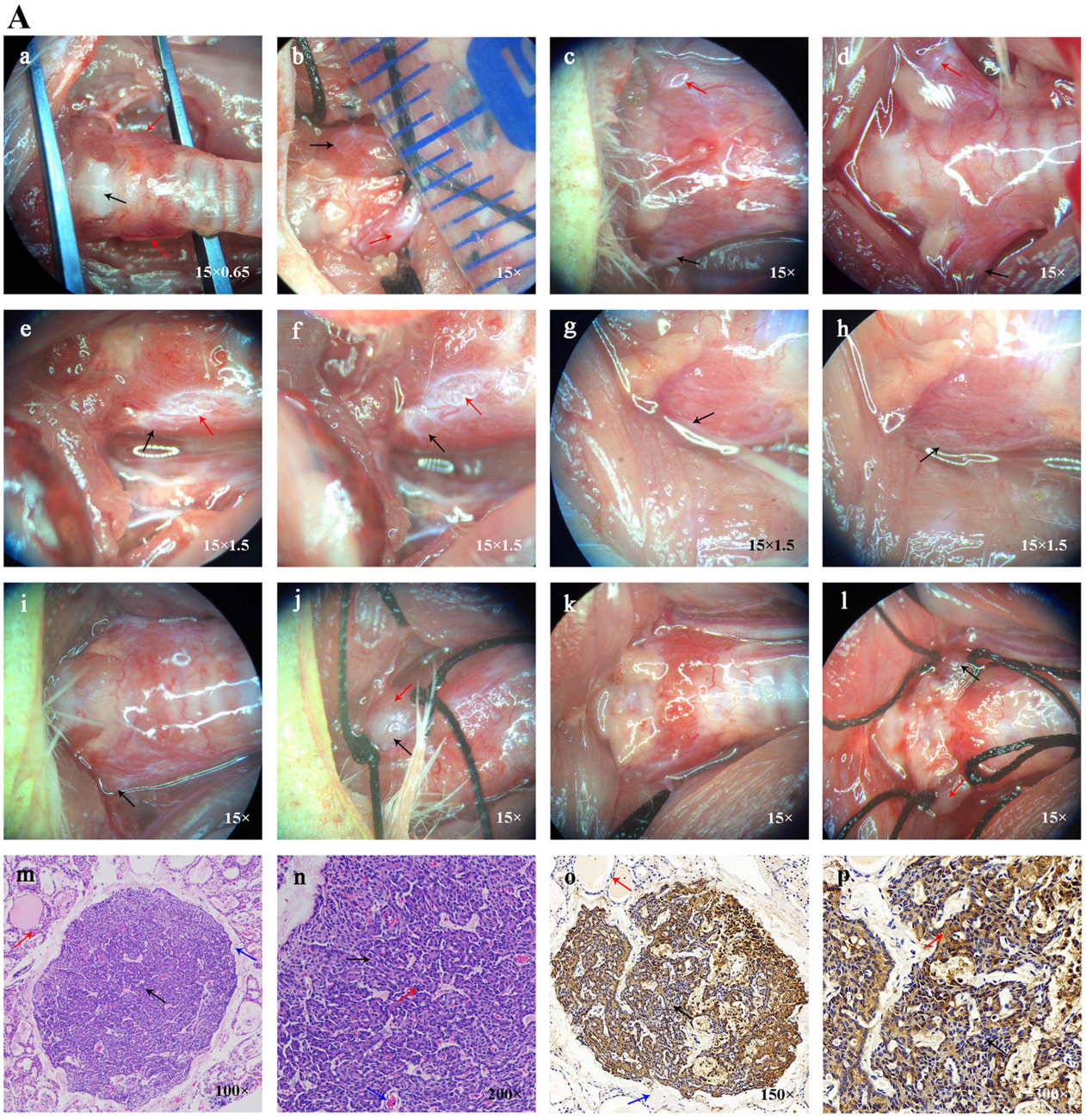
Statistical analysis

The data from 10 rats in which the PTGs were recognized and long-term survival was observed in each group were analyzed statistically. All statistical analyses were performed with IBM SPSS Statistics (version 23) (IBM, Armonk, NY, USA), and results were expressed as the mean ± SD. Repeated measures analysis of variance was used to determine the statistical significance of the indices between groups. Comparisons of the preoperative and postoperative data were performed using paired sample *t* tests. Evaluation of the indices was performed using receiver operating characteristic curves (ROC curves). Correlation analysis was performed using Pearson's correlation analysis, and a P value<0.05 was considered statistically significant.

Results

Anatomical and histological features

The parathyroid tissues of 56 rats were accurately searched for and identified based on the ischemia method under a stereomicroscope. Ultimately, 108 parathyroid tissues from 54 rats were identified; there was one rat in which the tissues could not be identified in both groups C and E, and there were no commonalities in the thyroid glands between these two rats (Fig. 1Aa), which made the total effective rate of PTG recognition for the 5 groups approximately 96% (54/56). The 108 parathyroid tissues were analyzed and categorized based on their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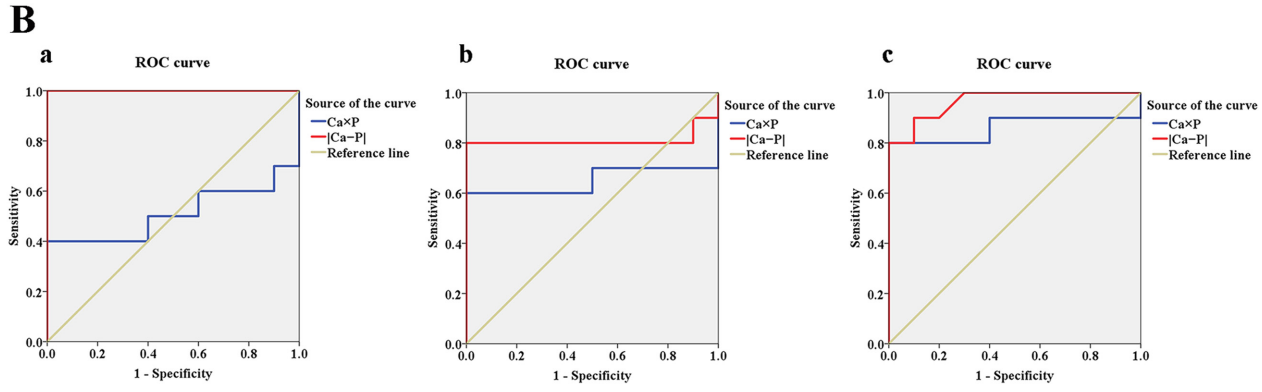
B
a
ROC curve
Sensitivity
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Source of the curve
Ca×P
|Ca−P|
Reference line
b
ROC curve
Sensitivity
1 - Specificity
Source of the curve
Ca×P
|Ca−P|
Reference line
c
ROC curve
Sensitivity
1 - Specificity
Source of the curve
Ca×P
|Ca−P|
Reference line

morphological characteristics and anatomical positions (Figs. 1Ab–p): The tissues were oval or round, approximately 1 mm in diameter (Fig. 1Ab), growing in and exposed to the surface of thyroid tissue, and sometimes protruded from the surfaces (Figs. 1Ac and d), and they were pale yellow or light red in color. They contained white capsules that were separated from thyroid tissues, with the capsules not being obvious or the capsules being distributed over a wide area in some cases, which made the parathyroid tissue difficult to identify by the visual method (Figs. 1Ae and f). Two tissues were identified in each rat, one each in the right and left lobes of the thyroid, and the tissues were found in the lateral part of the middle or superior poles of the right and left lobes of thyroid. However, the positions of bilateral parathyroid tissues were not consistent: the ventral side of the thyroid lobes accounted for 76% (82/108) of the tissues (Figs. 1Ac and d); the edge of the thyroid lobes, where the tissues could only be observed as complete oval or round tissues from the lateral edge of the thyroid and only incomplete tissues from the front, accounts for 19% (21/108) of the tissues (Figs. 1Ag and h); and the dorsal side of the thyroid lobes accounted for 5% (5/108) of the tissues (Figs. 1Ab, e, and f). Finally, the tissues were dense, and they sank after being placed into 0.9% sodium chloride solution.

The ligation blocking the blood supply to the suspected sites induced significant differences in color: the parathyroid showed a significant change in color, changing to white, while the thyroid did not significantly change in color (Figs. 1Ai–l). Different knotted suture positions have a definite influence on identification of the PTG, and the closer the knotted suture positions are to the PTG, the more obvious difference in color between the thyroid and PTG. The histopathological evaluations of the PTGs included H&E staining (Figs. 1Am and n) and immunohistochemical staining with rabbit anti-rat parathyroid cell-specific calcium-sensing receptor (CASR) polyclonal antibody [5] (Figs. 1Ao and p).

Postoperative symptoms and survival rate of rats

In this study, after excluding the two rats in which the PTGs were not recognized in groups C and E, 34 rats in groups C, D, and E were treated with TPTX based on the ischemia method, and they showed the main postoperative hypoparathyroidism symptoms of increased muscle stress and intermittent seizures, which were especially obvious when the rats were handled for blood draws [8, 19, 24]. Unfortunately, one rat treated with TPTX in group C died after the operation; the long-term survival rate of the rats with TPTX in groups C, D, and E was 97% (33/34). In groups A and B, 20 rats exhibited no symptoms of hypoparathyroidism, and all survived for the entire experimental period.

Results of statistical analysis

The biochemical indices in the groups are shown in Tables 1 to 5.

Effect of surgical resection of the PTGs on indices

Comparison of preoperative and postoperative indices within groups: There were no significant differences between the preoperative and postoperative indices, including the concentrations of serum calcium, phosphorus, and PTH (Table 1), Ca × P, and |Ca−P| (Table 2) in groups A, B, and A + B (Table 3). The postoperative serum calcium and PTH concentrations were significantly lower than the preoperative values in groups C, D, E, D + E (Table 4), and C + D + E (Table 3) ($P<0.05$), and the postoperative serum phosphorus concentrations and |Ca−P| values were significantly higher than the preoperative values in groups C, D, E, D + E, and C + D + E ($P<0.05$). The Ca × P

Fig. 1. Aa: the too small thyroid tissues (red arrow) on both sides of the thyroid cartilage (black arrow). Ab: the left parathyroid tissue on the ventral side (black arrow) and the right parathyroid tissue on the dorsal side after flipping to the ventral side (red arrow), both of which are about 1 mm in diameter. Ac and Ad: the four parathyroid tissues on the ventral side (black arrow and red arrow) and the left parathyroid tissues protrude from the surfaces of the thyroids (red arrow). Ae and Af: the right parathyroid tissue on the dorsal side after flipping to the ventral side in Af (black arrow) and the surrounding widely distributed capsules and suspected tissues in Af (red arrow). Ag and Ah: the parathyroid tissue on the edge side (black arrow). Ai: the right parathyroid tissue can be observed preliminarily (black arrow), and the left parathyroid tissue cannot be observed. Aj: the left parathyroid tissue on the edge (black arrow) and the surrounding thyroid tissue (red arrow) by the ischemia method. Ak: the parathyroid tissues cannot be observed. Al: the right parathyroid tissue on the edge (red arrow) and the left parathyroid tissue on the ventral side (black arrow) by the ischemia method. Am: the circular dense and deep dyed parathyroid tissue (black arrow), the peripheral lightly stained thyroid tissue (red arrow), and the fibrous tissue between them (blue arrow). An: the parathyroid cells arranged in clusters with clear cell boundaries, red stained cytoplasms (black arrow), blue stained nuclei (red arrow), and small blood vessels (blue arrow). Ao: the parathyroid tissue (black arrow), the thyroid tissue (red arrow), and the fibrous tissue (blue arrow). Ap: the positive expression of parathyroid tissue calcium-sensing receptor (CASR) protein in the tan area (red arrow) and a blue stained nucleus (black arrow). B: the analyses of the ROC curves of Ca×P and |Ca–P| at postoperative day 1 (Ba), day 14 (Bb), and day 21 (Bc) in group C.

Table 1. The preoperative and postoperative concentrations of calcium, phosphorus, and PTH in the serum of rats (mean ± SD)

Indices	Groups	Before operation	After operation (days)								
			1	3	7	14	21	28	35	42	49
Ca (mmol/l)	A (n=10)	2.52 ± 0.05	2.51 ± 0.03	2.53 ± 0.05	2.52 ± 0.04	2.52 ± 0.03	2.53 ± 0.04	2.5 ± 0.05	2.54 ± 0.06	2.52 ± 0.05	2.5 ± 0.06
	B (n=10)	2.54 ± 0.04	2.54 ± 0.05	2.53 ± 0.04	2.53 ± 0.04	2.51 ± 0.04	2.52 ± 0.05	2.52 ± 0.05	2.51 ± 0.05	2.5 ± 0.05	2.51 ± 0.07
	C (n=10)	2.53 ± 0.04	2.03 ± 0.15*#†	1.7 ± 0.19*#†	1.73 ± 0.25*#†	2.14 ± 0.12*#†	2.16 ± 0.1*#†	2.17 ± 0.1*#†	2.13 ± 0.11*#†	2.15 ± 0.11*#†	2.09 ± 0.1*#†
	D (n=10)	2.53 ± 0.03	1.92 ± 0.08*#†‡	1.48 ± 0.16*#†‡	1.39 ± 0.09*#†‡	1.94 ± 0.08*#†‡	2.01 ± 0.08*#†‡	1.89 ± 0.06*#†‡	1.95 ± 0.09*#†‡	1.96 ± 0.09*#†‡	1.93 ± 0.07*#†‡
	E (n=10)	2.55 ± 0.04	2.04 ± 0.12*#†¶	1.4 ± 0.15*#†‡	1.3 ± 0.09*#†‡	2.03 ± 0.07*#†‡¶	1.99 ± 0.08*#†‡	2.02 ± 0.07*#†‡¶	1.98 ± 0.08*#†‡	1.99 ± 0.08*#†‡	1.91 ± 0.08*#†‡
P (mmol/l)	A (n=10)	2.21 ± 0.13	2.29 ± 0.11	2.19 ± 0.11	2.28 ± 0.12	2.2 ± 0.12	2.23 ± 0.12	2.24 ± 0.12	2.21 ± 0.14	2.24 ± 0.14	2.27 ± 0.12
	B (n=10)	2.27 ± 0.08	2.26 ± 0.12	2.28 ± 0.11	2.27 ± 0.13	2.33 ± 0.13	2.3 ± 0.13	2.26 ± 0.15	2.31 ± 0.14	2.34 ± 0.12	2.27 ± 0.14
	C (n=10)	2.27 ± 0.03	2.84 ± 0.21*#†	3.24 ± 0.23*#†	3 ± 0.35*#†	2.79 ± 0.32*#†	2.79 ± 0.34*#†	2.92 ± 0.29*#†	2.96 ± 0.27*#†	2.92 ± 0.32*#†	3.01 ± 0.34*#†
	D (n=10)	2.26 ± 0.13	2.55 ± 0.26*#†‡	2.74 ± 0.11*#†‡	2.96 ± 0.23*#†	2.63 ± 0.27*#†	2.55 ± 0.12*#†‡	2.72 ± 0.25*#†‡	2.55 ± 0.19*#†‡	2.55 ± 0.2*#†‡	2.69 ± 0.18*#†‡
	E (n=10)	2.29 ± 0.12	2.76 ± 0.18*#†¶	2.93 ± 0.16*#†‡¶	3 ± 0.2*#†	2.53 ± 0.19*#†‡	2.6 ± 0.12*#†‡	2.59 ± 0.23*#†‡	2.66 ± 0.23*#†‡	2.6 ± 0.21*#†‡	2.61 ± 0.22*#†‡
PTH (ng/l)	A (n=10)	55.15 ± 5.88	55.99 ± 6.13	54.46 ± 6.41	54.94 ± 5.91	55.16 ± 5.75	56.65 ± 6.03	56.26 ± 5.81	55.7 ± 5.4	55.88 ± 5.02	55.98 ± 5.6
	B (n=10)	54.67 ± 6.2	54.54 ± 5.56	55.22 ± 5.93	56.23 ± 5.3	54.72 ± 5.69	55.16 ± 5.49	54.71 ± 5.38	55.24 ± 5.7	54.94 ± 5.2	56.44 ± 5.08
	C (n=10)	56.08 ± 5.29	38 ± 4.31*#†	24.58 ± 3.17*#†	20.42 ± 1.73*#†	19.99 ± 1.56*#†	20.75 ± 1.42*#†	20.47 ± 1.32*#†	20.27 ± 1.38*#†	20.4 ± 1.2*#†	20.86 ± 1.35*#†
	D (n=10)	56.57 ± 5.47	36.24 ± 4.18*#†	23.83 ± 2.49*#†	20.98 ± 1.84*#†	20.52 ± 1.23*#†	20.37 ± 1.42*#†	21 ± 1.37*#†	20.63 ± 1.26*#†	21 ± 1.48*#†	20.27 ± 1.3*#†
	E (n=10)	55.6 ± 5.71	38.26 ± 3.96*#†	25.05 ± 2.55*#†	21.26 ± 1.47*#†	20.88 ± 1.3*#†	20.69 ± 1.34*#†	21.17 ± 1.58*#†	21.07 ± 1.81*#†	21.11 ± 1.52*#†	20.58 ± 1.25*#†

*P<0.05, compared with before operation. #P<0.05, compared with group A. †P<0.05, compared with group B. ‡P<0.05, compared with group C. ¶P<0.05, compared with group D.

Table 2. The preoperative and postoperative Ca × P and |Ca−P| in the serum of rats (mean ± SD)

Indices	Groups	Before operation	After operation (days)								
			1	3	7	14	21	28	35	42	49
Ca×P ($mmol^2/l^2$)	A (n=10)	5.57 ± 0.3	5.75 ± 0.23	5.53 ± 0.21	5.75 ± 0.34	5.54 ± 0.31	5.63 ± 0.36	5.6 ± 0.25	5.61 ± 0.33	5.65 ± 0.29	5.67 ± 0.3
	B (n=10)	5.77 ± 0.25	5.74 ± 0.24	5.77 ± 0.28	5.75 ± 0.35	5.83 ± 0.34	5.8 ± 0.29	5.69 ± 0.35	5.8 ± 0.36	5.85 ± 0.28	5.7 ± 0.35
	C (n=10)	5.73 ± 0.1	5.74 ± 0.55	5.48 ± 0.51	5.12 ± 0.48*#†	5.96 ± 0.6#	5.99 ± 0.56#	6.31 ± 0.63*#†	6.29 ± 0.32*#†	6.23 ± 0.56*#†	6.27 ± 0.67*#†
	D (n=10)	5.71 ± 0.3	4.89 ± 0.47*#†‡	4.04 ± 0.42*#†‡	4.11 ± 0.23*#†‡	5.11 ± 0.61*#†‡	5.11 ± 0.15*#†‡	5.13 ± 0.38*#†‡	4.96 ± 0.27*#†‡	4.98 ± 0.3*#†‡	5.17 ± 0.31*#†‡
	E (n=10)	5.82 ± 0.3#	5.62 ± 0.31¶	4.08 ± 0.37*#†‡	3.9 ± 0.17*#†‡	5.12 ± 0.35*†‡	5.18 ± 0.37*#†‡	5.23 ± 0.44*†‡	5.25 ± 0.32*#†‡	5.16 ± 0.28*#†‡	4.98 ± 0.36*#†‡
|Ca−P| (mmol/l)	A (n=10)	0.31 ± 0.15	0.23 ± 0.13	0.34 ± 0.15	0.24 ± 0.12	0.32 ± 0.12	0.3 ± 0.11	0.26 ± 0.15	0.33 ± 0.17	0.28 ± 0.17	0.24 ± 0.15
	B (n=10)	0.26 ± 0.08	0.27 ± 0.15	0.24 ± 0.13	0.26 ± 0.13	0.18 ± 0.12	0.22 ± 0.16	0.26 ± 0.18	0.22 ± 0.11	0.2 ± 0.08	0.27 ± 0.13
	C (n=10)	0.26 ± 0.06	0.81 ± 0.28*#†	1.53 ± 0.37*#†	1.27 ± 0.57*#†	0.66 ± 0.38*#†	0.7 ± 0.29*#†	0.75 ± 0.33*#†	0.83 ± 0.37*#†	0.81 ± 0.29*#†	0.92 ± 0.39*#†
	D (n=10)	0.28 ± 0.13	0.63 ± 0.29*#†	1.26 ± 0.22*#†‡	1.57 ± 0.29*#†‡	0.68 ± 0.26*#†	0.54 ± 0.19*#†	0.82 ± 0.3*#†	0.61 ± 0.26*#†	0.59 ± 0.27*#†‡	0.76 ± 0.22*#†
	E (n=10)	0.26 ± 0.13	0.72 ± 0.26*#†	1.54 ± 0.27*#†¶	1.7 ± 0.27*#†‡	0.5 ± 0.23*†	0.61 ± 0.13*#†	0.57 ± 0.25*#†¶	0.68 ± 0.3*#†	0.64 ± 0.2*#†	0.7 ± 0.27*#†

*P<0.05, compared with before operation. #P<0.05, compared with group A. †P<0.05, compared with group B. ‡P<0.05, compared with group C. ¶P<0.05, compared with group D.

Table 3. The preoperative and postoperative indices of Ca, P, PTH, Ca × P, and |Ca−P| in the serum of rats in groups a + B and C + D + E (mean ± SD)

Indices	Groups	Before operation	After operation (days)								
			1	3	7	14	21	28	35	42	49
Ca (mmol/l)	A + B (n=20)	2.53 ± 0.04	2.53 ± 0.04	2.53 ± 0.04	2.52 ± 0.04	2.51 ± 0.03	2.53 ± 0.04	2.51 ± 0.05	2.52 ± 0.06	2.51 ± 0.05	2.51 ± 0.06
	C + D + E (n=30)	2.53 ± 0.04	2 ± 0.13*#	1.52 ± 0.21*#	1.48 ± 0.24*#	2.04 ± 0.12*#	2.05 ± 0.11*#	2.03 ± 0.14*#	2.02 ± 0.12*#	2.03 ± 0.12*#	1.98 ± 0.12*#
P (mmol/l)	A + B (n=20)	2.24 ± 0.11	2.28 ± 0.11	2.24 ± 0.12	2.28 ± 0.12	2.26 ± 0.14	2.26 ± 0.13	2.25 ± 0.13	2.26 ± 0.14	2.29 ± 0.13	2.27 ± 0.13
	C + D + E (n=30)	2.27 ± 0.1	2.72 ± 0.24*#	2.97 ± 0.27*#	2.99 ± 0.26*#	2.65 ± 0.28*#	2.65 ± 0.24*#	2.74 ± 0.29*#	2.72 ± 0.28*#	2.69 ± 0.29*#	2.77 ± 0.3*#
PTH (ng/l)	A + B (n=20)	54.91 ± 5.88	55.27 ± 5.74	54.84 ± 6.02	55.58 ± 5.5	54.94 ± 5.57	55.91 ± 5.67	55.48 ± 5.51	55.47 ± 5.41	55.41 ± 5	56.21 ± 5.21
	C + D + E (n=30)	56.08 ± 5.31	37.5 ± 4.11*#	24.48 ± 2.71*#	20.89 ± 1.67*#	20.47 ± 1.37*#	20.6 ± 1.35*#	20.88 ± 1.41*#	20.66 ± 1.49*#	20.84 ± 1.39*#	20.57 ± 1.28*#
Ca×P ($mmol^2/l^2$)	A + B (n=20)	5.67 ± 0.29	5.75 ± 0.23	5.65 ± 0.27	5.75 ± 0.33	5.68 ± 0.35	5.72 ± 0.33	5.64 ± 0.3	5.7 ± 0.35	5.75 ± 0.29	5.69 ± 0.32
	C + D + E (n=30)	5.75 ± 0.25	5.42 ± 0.58*#	4.53 ± 0.8*#	4.38 ± 0.62*#	5.4 ± 0.65*	5.43 ± 0.56#	5.55 ± 0.72	5.5 ± 0.65	5.46 ± 0.68#	5.47 ± 0.74
|Ca−P| (mmol/l)	A + B (n=20)	0.29 ± 0.12	0.25 ± 0.14	0.29 ± 0.15	0.25 ± 0.12	0.25 ± 0.14	0.26 ± 0.14	0.26 ± 0.16	0.27 ± 0.15	0.24 ± 0.13	0.25 ± 0.13
	C + D + E (n=30)	0.27 ± 0.11	0.72 ± 0.28*#	1.44 ± 0.31*#	1.51 ± 0.43*#	0.61 ± 0.3*#	0.62 ± 0.22*#	0.71 ± 0.3*#	0.7 ± 0.32*#	0.68 ± 0.27*#	0.79 ± 0.3*#

*P<0.05, compared with before operation. #P<0.05, compared with group A + B.

Table 4. The preoperative and postoperative indices of Ca, P, PTH, Ca × P, and |Ca−P| in the serum of rats in groups C and D + E (mean ± SD)

Indices	Groups	Before operation	After operation (days)								
			1	3	7	14	21	28	35	42	49
Ca (mmol/l)	C (n=10)	2.53 ± 0.04	2.03 ± 0.15*	1.7 ± 0.19*	1.73 ± 0.25*	2.14 ± 0.12*	2.16 ± 0.1*	2.17 ± 0.1*	2.13 ± 0.11*	2.15 ± 0.11*	2.09 ± 0.1*
	D + E (n=20)	2.54 ± 0.04	1.98 ± 0.11*	1.44 ± 0.16*#	1.35 ± 0.1*#	1.99 ± 0.09*#	2 ± 0.08*#	1.96 ± 0.09*#	1.96 ± 0.09*#	1.98 ± 0.08*#	1.92 ± 0.07*#
P (mmol/l)	C (n=10)	2.27 ± 0.03	2.84 ± 0.21*	3.24 ± 0.23*	3 ± 0.35*	2.79 ± 0.32*	2.79 ± 0.34*	2.92 ± 0.29*	2.96 ± 0.27*	2.92 ± 0.32*	3.01 ± 0.34*
	D + E (n=20)	2.27 ± 0.12	2.65 ± 0.24*	2.84 ± 0.17*#	2.98 ± 0.21*	2.58 ± 0.23*#	2.57 ± 0.12*	2.65 ± 0.25*#	2.61 ± 0.21*#	2.57 ± 0.2*#	2.65 ± 0.2*#
PTH (ng/l)	C (n=10)	56.08 ± 5.29	38 ± 4.31*	24.58 ± 3.17*	20.42 ± 1.73*	19.99 ± 1.56*	20.75 ± 1.42*	20.47 ± 1.32*	20.27 ± 1.38*	20.4 ± 1.2*	20.86 ± 1.35*
	D + E (n=20)	56.08 ± 5.46	37.25 ± 4.1*	24.44 ± 2.53*	21.12 ± 1.63*	20.7 ± 1.24*	20.53 ± 1.35*	21.09 ± 1.45*	20.85 ± 1.54*	21.05 ± 1.46*	20.42 ± 1.25*
Ca×P ($mmol^2/l^2$)	C (n=10)	5.73 ± 0.1	5.74 ± 0.55	5.48 ± 0.51	5.12 ± 0.48*	5.96 ± 0.6	5.99 ± 0.56	6.31 ± 0.63*	6.29 ± 0.32*	6.23 ± 0.56*	6.27 ± 0.67*
	D + E (n=20)	5.77 ± 0.3	5.26 ± 0.54*#	4.06 ± 0.39*#	4.01 ± 0.22*#	5.12 ± 0.48*#	5.15 ± 0.28*#	5.18 ± 0.4*#	5.1 ± 0.32*#	5.07 ± 0.3*#	5.07 ± 0.34*#
|Ca−P| (mmol/l)	C (n=10)	0.26 ± 0.06	0.81 ± 0.28*	1.53 ± 0.37*	1.27 ± 0.57*	0.66 ± 0.38*	0.7 ± 0.29*	0.75 ± 0.33*	0.83 ± 0.37*	0.81 ± 0.29*	0.92 ± 0.39*
	D + E (n=20)	0.27 ± 0.13	0.67 ± 0.27*	1.4 ± 0.28*	1.63 ± 0.28*	0.59 ± 0.26*	0.57 ± 0.16*	0.7 ± 0.3*	0.64 ± 0.28*	0.61 ± 0.23*#	0.73 ± 0.24*

*P<0.05, compared with before operation. #P<0.05, compared with group C.

Table 5. Pearson's correlations of the preoperative and postoperative calcium and phosphorus concentrations in the serum of rats

Groups	Before operation		After operation (days)															
			1		3		7		14		21		28		35		42		49	
	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P	Correlation coefficient	P
A (n=10)	−0.307	0.388	−0.611	0.061	−0.826**	0.003	0.187	0.605	0.06	0.87	0.478	0.162	−0.562	0.091	−0.386	0.27	−0.667*	0.035	−0.274	0.443
B (n=10)	0.221	0.54	−0.672*	0.033	−0.197	0.586	0.087	0.812	0.216	0.549	−0.537	0.11	−0.388	0.268	−0.044	0.905	−0.364	0.301	−0.224	0.534
C (n=10)	−0.418	0.23	−0.173	0.632	−0.553	0.097	−0.812**	0.004	−0.461	0.18	−0.801**	0.005	−0.248	0.49	−0.912**	0	−0.686*	0.028	−0.39	0.265
D (n=10)	−0.423	0.223	−0.317	0.373	−0.298	0.403	−0.684*	0.029	0.22	0.541	−0.781**	0.008	−0.749*	0.013	−0.629	0.051	−0.640*	0.046	−0.467	0.173
E (n=10)	−0.067	0.855	−0.603	0.065	−0.526	0.119	−0.779**	0.008	−0.466	0.174	0.31	0.383	−0.411	0.238	−0.812**	0.004	−0.904**	0	−0.552	0.098
D + E (n=20)	−0.198	0.402	−0.082	0.731	−0.483*	0.031	−0.687**	0.001	−0.15	0.529	−0.241	0.307	−0.573**	0.008	−0.628**	0.003	−0.713**	0	−0.478*	0.033

*P<0.05, considered statistically significant; **P<0.01, considered statistically evidently significant.

values mostly showed no significant differences from the preoperative value in the early postoperative period at 21 days and were significantly higher than the preoperative value in the late postoperative period in group C ($P<0.05$). The postoperative Ca × P values were significantly lower than the preoperative values in groups D, E, and D + E for the majority of the time points ($P<0.05$). The Ca × P values were significantly lower than the preoperative value in the early postoperative period from day 1 to day 14 ($P<0.05$) and showed no significant differences from the preoperative value in the late postoperative period in group C + D + E.

Analyses of the ROC curves for Ca × P and |Ca−P| at postoperative day 1, day 14, and day 21 compared with the preoperative indices in group C were shown in Figs. 1Ba–c, respectively. The area under the curve (AUC) of |Ca−P| was 1 ($P<0.05$), which was bigger than that of Ca × P (0.51), at postoperative day 1, it was 0.81 ($P<0.05$), which was bigger than that of Ca × P (0.65), at postoperative day 14, and it was 0.965 ($P<0.05$), which was bigger than that of Ca × P (0.86), at postoperative day 21. This shows that |Ca−P| was superior to Ca × P in terms of reflecting surgical resection of the PTGs in group C.

Concerning the trends in changes of the indices, the preoperative and postoperative indices remained stable in groups A, B, and A + B, while the postoperative serum calcium concentrations and Ca × P values in groups C, D, E, D + E, and C + D + E decreased to their lowest levels at postoperative day 3 or day 7 and then increased to stable levels at postoperative day 14 or day 28. The postoperative serum phosphorus concentrations and |Ca−P| values in groups C, D, E, D + E, and C + D + E increased to their highest levels at postoperative day 3 or day 7 and then decreased to a stable level at postoperative day 14. The postoperative serum PTH concentrations in groups C, D, E, D + E, and C + D + E decreased to their lowest levels at postoperative day 7 and then remained stable.

As shown in Table 5, there was no significant correlation between the preoperative serum calcium and phosphorus concentrations in the groups. There were significant negative correlations at postoperative day 3 ($P<0.01$) and at postoperative day 42 ($P<0.05$) in group A, at postoperative day 1 ($P<0.05$) in group B, at postoperative days 7, 21, and 35 ($P<0.01$) and at postoperative day 42 ($P<0.05$) in group C, at postoperative days 7, 28, and 42 ($P<0.05$) and at postoperative day 21 ($P<0.01$) in group D, at postoperative days 7, 35, and 42 in group E ($P<0.01$), and at the postoperative days 3 and 49 ($P<0.05$) and postoperative days 7, 28, 35, and 42 ($P<0.01$) in group D + E.

Regarding changes in the discretizations and stabilities of the serum calcium and phosphorus concentrations, the standard deviations of serum calcium concentrations were lower than those of serum phosphorus concentrations at the same time points (Table 1). There were no significant changes in preoperative and postoperative standard deviations of the two indices in groups A and B, whereas the postoperative standard deviations in groups C, D, and E were significantly larger than those in the preoperative period.

Comparison between groups A and C for rats drinking tap water: As shown in Tables 1 and 2, there were no significant differences in the preoperative indices between groups A and C. The postoperative serum calcium and PTH concentrations in group C were significantly lower than those in group A ($P<0.05$), and the postoperative serum phosphorus concentrations and |Ca−P| values in group C were significantly higher than those in group A ($P<0.05$). The postoperative Ca × P values in group C were significantly higher than those in group A until postoperative day 14 ($P<0.05$). The postoperative negative correlation between the serum calcium and phosphorus concentrations in group C was higher than that in group A (Table 3). There was little difference in the standard deviations of the preoperative serum calcium concentrations between groups A and C, and the standard deviation of the preoperative serum phosphorus concentrations in group A was greater than that in group C. However, the postoperative standard deviations of the two indices in group C were significantly greater than those in group A.

Comparison between groups B and D for rats drinking distilled water and between groups B and E after operation for rats drinking distilled water: As shown in Tables 1 and 2, there were no significant differences in the preoperative indices between groups B and D. The postoperative serum calcium and PTH concentrations in groups D and E were significantly lower than those in group B ($P<0.05$), but the postoperative serum phosphorus concentrations and |Ca−P| values in groups D and E were significantly higher than those in group B ($P<0.05$). The postoperative Ca × P values in groups D and E were significantly lower than those in group B for the majority of the time points ($P<0.05$). The postoperative negative correlations between the serum calcium and phosphorus concentrations in groups D and E were higher

than that in group B (Table 5). There was little difference in the standard deviations of the preoperative serum calcium and phosphorus concentrations between groups B and D, whereas the postoperative standard deviations in groups D and E were significantly greater than those in group B for the majority of the time points.

Comparison between group A + B and group C + D + E regardless of water type: To compare the effects of surgical removal of the PTGs and varying types of water intake on the indices, group A + B, which contained rats without TPTX, and group C + D + E, which contained rats with TPTX, were compared regardless of water type. As shown in Table 3, there were no significant differences in the preoperative indices between group A + B and group C + D + E. The postoperative serum calcium and PTH concentrations in group C + D + E were significantly lower than those in group A + B ($P<0.05$), and the postoperative serum phosphorus concentrations in group C + D + E were significantly higher than those in group A + B ($P<0.05$).

Effect of rats drinking different types of water

Comparison between groups A and B without the removal of the PTGs in rats that drank different types of water: As shown in Table 1 and 2, there were no significant differences in the preoperative and postoperative indices and the standard deviations of the serum calcium and phosphorus concentrations between groups A and B. There were no significant correlations between the serum calcium and phosphorus concentrations in groups A and B for the majority of the time points (Table 5).

Comparison between group C and groups D, E, and D + E with removal of the PTGs in rats that drank different types of water after operation: As shown in Table 1, 2, and 4, the postoperative serum calcium and phosphorus concentrations and Ca × P values in groups D, E, and D+E were significantly lower than those in group C for the majority of the time points ($P<0.05$). There were no significant differences in the postoperative PTH concentrations and |Ca−P| values between group C and groups D, E, and D + E for the majority of the time points ($P<0.05$). As described above, the change trends of the same postoperative indices in group C and groups D, E, and D + E were consistent. There were no significant differences in the negative correlations between the postoperative serum calcium and phosphorus concentrations between group C and groups D and E, and the negative correlations in group D + E were significantly higher than those in group C (Table 5). The standard deviations of the postoperative serum calcium and phosphorus concentrations in group C were greater than those in groups D, E, and D + E for the majority of the time points.

Discussion

Calcium-phosphorus abnormalities are the basis for CKD-MBD occurrence, primarily hypocalcemia, hyperphosphatemia, and increased Ca × P [3]. The TPTX rat models established in this experiment also exhibit hypocalcemia and hyperphosphatemia, so they can also be used as animal models and to support techniques for studies of the calcium and phosphorus metabolism disorders in CKD-MBD.

Due to the variations in the anatomic site and appearance of the PTG [10], accurate intraoperative identification and resection of the PTG are the keys to PTX in the clinic. Identification methods include the visual method, float method, methylene blue staining [6], 99Tcm-MIBI scintigraphy [13], and intraoperative pathological examination. For PTX, accurate preoperative positioning of parathyroid tissues is recommended, and the intraoperative visual method has some limitations, as Abboud *et al.* reported 252 cases of surgery using the visual method to identify PTGs, 1, 2, 3, or 4 PTGs were identified in 2, 7, 41, and 202 cases, respectively [1]. After accurate identification, surgeons resect the parathyroid tissues completely along with the surrounding lymphatic and adipose tissues. The rat TPTX models also face the same problems. Successful establishment of a TPTX model with simple microscopic visual recognition and direct resection of suspected PTGs is difficult to guarantee, and it is easy to remove a large amount of thyroid tissue and injure important vessels and nerves, leading to complications.

In this study, we examined the parathyroid tissues of 56 rats by the ischemia method under a stereomicroscope, and 108 parathyroid tissues from 54 rats were identified. The anatomical locations of PTGs are not fixed and do not correspond bilaterally, and PTGs may be located in the lateral parts of the middle or superior poles of the lobes of the thyroid, including the ventral side, lateral edge, and dorsal side, with accounted for 76%, 19%, and 5% of the locations in the present study, respectively. However, there were two rats in which the bilateral parathyroid tissues could not be identified accurately in the present study, and we believe that their PTG tissues might have been hidden in the thyroid tis-

sues due to their individual differences in terms of the growth of the thyroid and PTG tissues.

In the present study, rat TPTX models were successfully established by the ischemia method with obvious postoperative symptoms of hypoparathyroidism, including increased muscle stress and intermittent seizures [8, 19, 24]. After removal of the PTGs, the serum PTH concentrations decreased significantly, and the induced calcium and phosphorus metabolism disorders significantly decreased serum calcium concentrations and increased serum phosphorus concentrations. The effects of a lack of regulation of PTH on serum calcium and phosphorus concentrations were greater than those of drinking the different types of water, as shown by the comparison between groups A + B and C + D + E. After removal of the PTGs, the serum calcium and phosphorus concentrations gradually returned to stable levels at postoperative 14 days, which indicates that the time point of early postoperative day 14 is appropriate for the transplantation of PTGs in hypoparathyroidism rats. The postoperative serum PTH concentrations in rats with TPTX were high, even when they decreased to their lowest levels, and we considered that the measurements of the postoperative serum levels of PTH might have been affected by parathyroid hormone-related protein (PTHrp), which has amino acid sequences and conformations that are similar to those of PTH. PTHrp is expressed in many tissues and organs such as bone, and it acts on bone, the kidney, and other organs [23].

The synchronous change trends of the postoperative serum calcium and phosphorus concentrations were generally opposite. In theory, there are negative correlations between the two indices, and Ca × P, which represents an index for an osteogenesis unit of measurement, is 35–40 mg^2/dl^2 in normal adults [24]. When Ca × P<35, bone calcification is hindered, which affects osteogenesis and causes rickets. When Ca × P>40, calcium and phosphorus deposit in bone tissues [22]. When Ca × P>60–70, calcium-phosphate crystals can deposit in blood vessels and soft tissues [8]. In this study, the serum calcium and phosphorus concentrations in the groups did not show significant negative correlations under the regulation of PTH at the majority of time points, probably due to the limited number of rats. But the negative correlations significantly increased after removal of the PTGs, and this might be explained as follows: when one index of the serum calcium and phosphorus concentrations changed, the other index might have remained stable under the regulation of PTH but might have exhibited a significant inverse change after removal of the PTGs, leading to more obvious opposite change trends between the two indices. Serum phosphorus concentrations were relatively more unstable than serum calcium concentrations at the same time points, which showed that serum phosphorus concentrations were not sensitive or specific indicators of phosphorus balance in the body [8], and the fluctuations of indices increased without the regulation of PTH.

Regarding the effects of rats drinking different types of water on the serum indices, when the PTGs were not removed, the differences in calcium and phosphorus concentrations in water were not sufficient to create the significant differences in serum indices or the correlations of serum calcium and phosphorus concentrations on the basis of feeding rats solid forage. But when the PTGs were removed, the effects became more obvious. Drinking tap water made the serum calcium and phosphorus concentrations and the discretizations of the two indices of rats larger than those of rats drinking distilled water; this may be because without the regulation of PTH, rats drinking tap water were not limited in the intake of calcium and phosphorus in water. However, drinking different types of water did not affect the change trends of the same postoperative indices or the negative correlations between serum calcium and phosphorus concentrations in groups without the regulation of PTH.

Considering that instabilities of the serum calcium and phosphorus concentrations individually and the application of clinical indices, the two serum indices were combined to the Ca × P and |Ca−P| values. The following four findings observations were made: (1) Ca × P reflected the actual effects of removal of the PTGs in just some of the groups. After removal of the PTGs, Ca × P showed change trends similar to those of the serum calcium concentrations. (2) After the removal of the PTGs, Ca × P accurately reflected the effect of varying the types of water intake in rats and was superior to the serum phosphorus concentration. (3) |Ca−P| accurately reflected the effect of removal of the PTGs and was superior to Ca × P, and after removal of the PTGs, |Ca−P| showed change trends similar to those of serum phosphorus concentrations. (4) After the removal of the PTGs, |Ca−P| did not reflect the effect of varying the types of water intake in rats. For the four findings observations, we found that surgical removal of the PTGs had divergent effects on the serum calcium and phosphorus concentrations. The serum calcium concentrations were higher

than the serum phosphorus concentrations before the TPTX operation, but after removal of the PTGs, they were lower than the serum phosphorus concentrations, and the differences between the two indices as |Ca−P| increased, which made the postoperative Ca × P inevitably appear to show no significant differences from the preoperative Ca × P. After removal of the PTGs, drinking different types of water made the two indices change in the same direction, with the changes in |Ca−P| being weaker and those in Ca × P being stronger.

The ischemia method provides significant advantages in establishing a rat TPTX model: 1) It is conducive to intraoperative PTG recognition with an effective recognition rate of approximately 96% (54/56). 2) It is conducive to reducing bleeding compared with the direct removal of the PTGs, which can easily cause bleeding due to the rich thyroid blood supply [24]. 3) It is conducive to improving the success rate of establishing models with accurate recognition of the PTGs and residual tissue, with the rate of establishing models being 100% (34/34) in groups C, D, and E in the present study, excluding two rats without TPTX. Direct removal of the PTGs can cause serious bleeding, which makes it difficult to find residual tissues and may lead to failure of model establishment. 4) Saving the intact thyroid reduces the impact of hypothyroidism and calcitonin (CT) fluctuations on the indices. To ensure no residual PTG tissue is present, direct resection is bound to remove a large amount of thyroid tissue and is likely to cause hypothyroidism, with symptoms including reduced eating and drinking, weight loss, and even death [8]. CT was secreted by thyroid parafollicular cells, and it mainly reduces the serum calcium and phosphorus concentrations [8]. 5) Finally, the ischemia method is conducive to increasing the postoperative long-term survival rate of rats by reducing the occurrence of hypothyroidism, blood loss, and injury of the recurrent laryngeal nerve [8, 24].

The visual method has some limitations in identifying the PTG in rats when the anatomical features of the parathyroid tissues are not obvious. Compared with the visual method, the ischemia method can effectively identify parathyroid tissues, especially those that cannot be identified by the visual method alone when the tissues do not protrude from the surface of the thyroid, do not exhibit a significant color difference compared with the thyroid tissues, do not have an obvious white parathyroid capsule, or have widely distributed capsules. Some experts have used the 5-aminolevulinic acid (5-ALA) fluorescence-guide identification method to identify PTGs in rats, but 5-ALA could cause phototoxic effects when activated, e.g., by sunlight [11]. We also found that clamping the upper thyroid artery to block one side of the thyroid lobe blood supply to identify a PTG also made use of the same ischemic principle but had far more negative effects, including a wide range of ischemic and hypoxic injuries to the thyroid and damage to the small vessels. Additionally, the effect on identification was difficult to guarantee in the case of clipping sites relatively far away from the PTG, and it required an extended period of time to observe the subtle changes in color after tissue ischemia. This method was also faced with the negative effects of direct removal of the PTG.

Conclusion

The ischemia method had advantages for accurate identification and excision of the PTGs of rats and was beneficial for the establishment of rat TPTX models. The effects of regulation of PTH on indices were greater than those of rats drinking different types of water. After the removal of the PTGs, the postoperative indices showed significant changes, with increased negative correlations between serum calcium and phosphorus concentrations and fluctuations of the two indices, and the effects of rats drinking different types of water on the indices increased, with significant differences between groups and the increased fluctuations of the indices. |Ca−P| accurately reflected the effect of removal of the PTGs and was superior to Ca × P, and Ca × P accurately reflected the effect of varying the types of water intake in rats and was superior to the serum phosphorus concentration and |Ca−P|.

Acknowledgments

Haoran Yuan would like to thank Dr. Yinghua Li, Dr. Lin Yang, and Dr. Yongzhao Shi for their helps in the data analysis and in the editing and revising of this article. The authors declare that they have no conflicts of interests regarding publication of this article. The study was funded by the mammary key specialist construction project of the Fifth People's Hospital of Shanghai affiliated with Fudan University (Code: 2013 WYZDZK05) and a project of the Shanghai Minhang District Health Bureau (Code: 2012 MW03).

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