Role of Ca$^{2+}$ in Inhibiting Ischemia-Induced Apoptosis of Parathyroid Gland Cells in New Zealand White Rabbits

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Source of support: The study was supported by National Natural Science Foundation of China (Grant No. 8176030147) and the Scientific Research Fund of the Yunnan Education Department (Grant No. 20191248)

Background: Hypoparathyroidism is a common complication after thyroidectomy. Calcium supplementation can relieve these symptoms, but it is not clear whether it can protect the parathyroid glands. This study aimed to verify whether Ca$^{2+}$ inhibits the apoptosis of parathyroid cells following ischemic injury.

Material/Methods: A rabbit model of parathyroid gland ischemic injury was established. The blood calcium concentrations were measured by colorimetry. The parathyroid hormone (PTH) levels were measured by enzyme-linked immunosorbent assay (ELISA). The parathyroid tissues were observed by hematoxylin and eosin (H&E) staining and the TdT-mediated dUTP nick-end labeling (TUNEL) assay. Western blotting was used to quantify the levels of the following proteins: caspase-3 and p38 MAPK.

Results: This study demonstrates that apoptosis can be a part of the pathological changes associated with parathyroid ischemic injury. Calcium supplementation inhibited the apoptosis of parathyroid cells following ischemic injury. There were no significant differences among the serum calcium levels from the Sham operation (Sham), the Control group (CG), or the Calcium supplementation group (CSG) after 24 h, 72 h, and 168 h of treatment. PTH levels in the CG were significantly higher than in the CSG at 24 h and 72 h after treatments. The apoptosis rate of parathyroid cells from rabbits in the CSG was significantly lower than that of those from rabbits in the CG at 24 h and 72 h after the treatment. Calcium supplementation inhibited p38 MAPK and caspase-3 expression.

Conclusions: This study demonstrates that calcium supplementation inhibited the apoptosis of parathyroid cells following ischemic injury.

MeSH Keywords: Apoptosis • Calcium • Ischemia • p38 Mitogen-Activated Protein Kinases • Parathyroid Glands

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/920546
Background

Thyroidectomy, as the main treatment for thyroid cancer, carries high risks of transient or permanent hypoparathyroidism [1,2]. The main function of the parathyroid gland is to secrete parathyroid hormone (PTH) and participate in regulation of calcium and phosphorus metabolism in vivo [3,4]. At present, it is considered that in situ exposure and protection of parathyroid glands during surgery is an effective way to avoid permanent hypoparathyroidism [5–8]. However, the parathyroid glands are small and closely connected to the thyroid gland and central lymph nodes [9,10], and in situ protection can cause mechanical and thermal damage to the parathyroid glands. The average half-life of PTH is only 17.4 min [11]. After parathyroid injury, PTH levels rapidly decrease, resulting in decreased blood calcium and causing hypocalcemia [12].

After parathyroid injury, primary PTH secretion decreases, which leads to a secondary decrease in serum calcium level. Hypocalcemia stimulates the synthesis and secretion of PTH by damaged parathyroid cells, thereby maintaining normal serum calcium levels. Calcium supplementation after thyroidectomy can alleviate hypocalcemia [13], but it is unclear whether calcium supplementation has protective effects on the parathyroid gland. Therapeutic or predictive calcium supplementation is still available, and the peak of hypocalcemia occurs 24 h after surgery [14], which is also the most severe period of parathyroid injury. Hypercalcemia inhibits the secretion of PTH by the parathyroid gland, but does not induce apoptosis [15,16]. We speculate that Ca$^{2+}$ inhibits the apoptosis of parathyroid gland cells following ischemic injury. We tested our hypothesis by analyzing the parathyroid cells of a rabbit model of parathyroid gland ischemic injury following calcium supplementation.

Material and Methods

Animals

Male New Zealand white rabbits (3.0–3.5 kg, n=54) were purchased from the Laboratory Animal Center of Kunming Medical University. They were housed at a constant temperature, with an identical photoperiod (light from 07:00 to 19:00). All surgical and experimental procedures were approved by the Ethics Committee of Kunming Medical University (Protocol Number: kmnu2019056) and carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Establishment of the unilateral parathyroid ischemic injury model

Venous indwelling needles were implanted and fixed into the ears of all the animals (n=54). The rabbits were anesthetized via injection of 3% sodium pentobarbital (1.0 ml/kg BW) into the auricular vein. In the Sham operation group (Sham group), only the bilateral inferior parathyroid glands were exposed. In the Control group (CG) and Calcium supplementation group (CSG), the unilateral rabbit inferior parathyroid glands were randomly selected and we only ligated the main blood supply vessels. Sodium pentobarbital (3%) was purchased from the Animal Laboratory Center of Kunming Medical University.

Grouping

All animals were treated as follows: within 30 min after surgery, the total amount of each fluid infused was 80 ml/kg BW per day; calcium gluconate solution (0.0167 g/ml/kg WB) was administered to the rabbits from the CSG for 24 h, 72 h, and 168 h (each group n=6); the rabbits in the CG and Sham group were administered 10% glucose solution for 24 h, 36 h, and 168 h (each group n=6). The fluids were administered using an infusion pump, which was filled up in 6 h. Calcium gluconate (1 g/10 ml) (Sichuan Medco Pharmaceutical Stock Co., Sichuan, China) and 10% glucose (Sichuan Medco Pharmaceutical Stock Co., Sichuan, China) solutions for injection were purchased from the First Affiliated Hospital of the Kunming Medical University. After the rehydration was completed, the rabbits were sacrificed with sodium pentobarbital (10%) and their parathyroid glands were harvested.

Detection of free calcium in the serum

In this study, the serum free calcium levels were measured by an enzyme labeling assay using a serum free calcium concentration detection kit (BC0720, Solarbio, Beijing, China); the absorbance of the samples was measured at a continuous wavelength (SPECTRAMAX190, Molecular Devices, CA, USA).

Detection of PTH

In this experiment, the PTH levels were assessed using a Rabbit PTH ELISA kit (LS-F24201, LifeSpan BioSciences, Inc., USA); the absorbance of the samples was measured using a Microplate Reader (SPECTRAMAX190, Molecular Devices, CA, USA).

Hematoxylin and eosin (H&E) staining

After subjecting the parathyroid tissues to paraffin embedding, the paraffin-embedded parathyroid tissue sections were stained with H&E. The parathyroid tissues were first immobilized in 10% formalin and then embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 5 μm and
dewaxed. H&E staining was then performed. The H&E-stained sections were analyzed by 2 senior pathologists.

**TUNEL assay**

The paraffin-embedded parathyroid tissue sections were subjected to the TdT-mediated dUTP nick-end labeling (TUNEL) analysis. TUNEL staining was performed using the In-Situ Cell Death Detection Kit (Roche, Germany); the stained cells were analyzed using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). Three fields of vision (200×) were randomly selected. We used Image J software (National Institutes of Health, Maryland, US) to quantify the number of apoptotic cells and total number of TUNEL-stained cells. The apoptosis rate was then calculated and the results are expressed as the percentage of number of apoptotic cells/total number of cells.

**Western blotting analysis**

Proteins were extracted from the thyroid tissues and separated by SDS-PAGE according to standard protocols; the resulting protein bands were transferred onto PVDF membranes.
for immunoblotting. Caspase-3 (Abcam, Cambridgeshire, UK, 1/2000)- and p38 MAPK (GeneTex, Southern California, USA, 1/1500)-specific antibodies were used for this analysis.

**Statistical analysis**

SPSS 24.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The data are expressed as the means±standard errors of the means (SEMs). The paired t test was used to compare the differences of all parameters between the control and the experimental groups before and after the treatment, and one-way ANOVA was used to determine the differences between the 2 groups. Differences with P<0.05 were considered statistically significant.

**Figure 2.** Effect of calcium supplementation on the PTH levels. Before: Preoperative PTH level, after: Postoperative PTH level. (A) Serum PTH levels in rabbits from the Sham operation group, control group, and calcium supplementation group 24 h, 72 h, and 168 h before and after the treatment. (B) The serum PTH levels in rabbits from the Sham operation group, control group, and calcium supplementation group were measured at the 24-h, 72-h, and 168-h time points. The data are expressed as the means±standard errors of the means (SEMs). * P<0.05, ** P<0.01, *** P<0.001.
Results

Changes of the serum calcium levels before and after parathyroid gland ischemic injury

The rabbits from the Sham group, CG, and CSG were administered the same amount of fluids intravenously. To evaluate the effects of Ca^{2+} supplementation on the serum free calcium levels in the animals, we measured the serum free calcium levels in the rabbits from each group before and after treatment. The results showed that there were no significant differences between the serum free calcium levels in rabbits from these 3 groups after 24, 72, and 168 h of the treatments (Figure 1; \( P>0.05 \) in all cases; each group \( n=6 \)), indicating that the serum free calcium levels were the same before and after the treatment.

A

24 h

72 h

168 h

Sham

CG

CSG

B

24 h

72 h

168 h

Sham

CG

CSG

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

© Med Sci Monit, 2020; 26: e920546
Changes in PTH before and after parathyroid gland ischemic injury

Because no difference was observed between the serum calcium levels before and after the treatment, the PTH levels before and after the treatment were measured. The PTH levels in rabbits from the Sham group showed no significant difference before and after 24 h, 72 h, and 168 h of the treatments (Figure 2A; top). The PTH levels in rabbits from the CG after 24 h and 72 h of treatment were significantly higher than those before 24 h and 72 h of the treatments, respectively (CG, 24 h after treatment: 29.34±10.20 vs. CG, 24 h before treatment: 23.07±7.95; *P<0.05; and CG, 72 h after treatment: 33.82±5.19 vs. CG, 72 h before treatment: 26.05±5.16; **P<0.01; Figure 2A, middle panel; each group n=6). There was no significant difference in PTH levels between before and after treatment in the Control group at 168 h and in the Calcium supplementation group at 24 h and 168 h before treatment (all *P>0.05; Figure 2, middle and lower panel; each group n=6).

Assessment by H&E staining of the pathological changes in ischemic parathyroid glands following Ca²⁺ supplementation

Analysis of the H&E-stained sections of the parathyroid gland samples obtained from rabbits in the CSG and CG at 24 h, 72 h, and 168 h after the treatments showed that the nuclear structure of the parathyroid cells was intact, the nested structure of the gland still existed, and the necrosis in the cells was not obvious (Figure 3A). Fibrosis of the parathyroid tissues began to appear at 24 h, the area of fibrosis had increased at 72 h, and was notable at 168 h.

Assessment of the apoptosis in ischemic parathyroid glands following Ca²⁺ supplementation by TUNEL staining

Apoptosis of parathyroid gland cells following ischemic injury was assessed by TUNEL analysis. The green fluorescence indicates the apoptotic cells and the blue fluorescence indicates the normal cells. There was no significant difference between the apoptotic rate of parathyroid cells of rabbits from the Sham group at 24 h, 72 h, and 168 h after the treatments (all *P>0.05; Figure 3C, left; each group n=6). There was no significant difference between the apoptotic rate of parathyroid cells of rabbits from the CG at 24 h, 72 h, and 168 h after the treatments (P>0.05; Figure 3C, middle; each
There was no significant difference between the apoptotic rate of parathyroid cells of rabbits from the CSG at 24 h, 72 h, and 168 h after the treatments ($P>0.05$; Figure 3C, right; each group $n=6$). The apoptotic rate of parathyroid cells of rabbits from the CG was higher than that of the parathyroid cells of rabbits from the CSG ($CG, 24 \text{ h}: 24.66\pm4.88\% \text{ vs. } CSG, 24 \text{ h}: 10.75\pm5.03\%; P<0.001$; Figure 3D, left; and $CG, 72 \text{ h}: 23.5383\pm5.42\% \text{ vs. } CSG, 72 \text{ h}: 14.76\pm4.25\%; P<0.05$; Figure 3D, middle; each group $n=6$). There was no significant difference between the apoptotic rate of parathyroid cells of rabbits from the CG and CSG at 168 h after the treatments ($P>0.05$; Figure 3D, right; each group $n=6$). Overall, these results indicated that the inhibitory effect of $Ca^{2+}$ on the ischemic injury-induced apoptosis of parathyroid cells began at 24 h time and lasted until 72 h. There was no difference between the apoptotic rates of parathyroid cells of rabbits from the CG and CSG on the 7th day.

**Ca$^{2+}$ supplementation inhibits the expression of caspase-3 and p38 MAPK in parathyroid cells following ischemic injury**

When the level of beta-actin was set at 1.00, the levels of p38 MAPK in the parathyroid cells from rabbits in the Sham group, CG, and CSG at 24 h after the treatments were 0.39$\pm$0.04, 0.92$\pm$0.04, and 1.16$\pm$0.12, respectively (Figure 4A). The levels of p38 MAPK in the parathyroid cells from the rabbits in the Sham group, CG, and CSG at 72 h after the treatments were 0.57$\pm$0.13, 1.36$\pm$0.07, and 1.39$\pm$0.12, respectively (Figure 4B). The levels of p38 MAPK in the parathyroid cells from the rabbits in the Sham group, CG, and CSG at 168 h after the treatments were 0.51$\pm$0.09, 1.11$\pm$0.10, and 0.83$\pm$0.08, respectively (Figure 4B). These results showed that the expression of p38 MAPK in the parathyroid cells had decreased by 168 h after $Ca^{2+}$ supplementation. The data are expressed as the means$\pm$standard errors of the means (SEMs).

* $P<0.05$ compared to the Sham group. ** $P<0.01$ compared to the Sham group. *** $P<0.001$ compared to the Sham group. # $P<0.05$ compared to the Control group. ## $P<0.01$ compared to the Control group.
Ca\textsuperscript{2+} supplementation (P<0.05), but had increased by 24 h (P<0.05); there was no difference between the p38 MAPK levels in the presence and absence of Ca\textsuperscript{2+} supplementation at 72 h (P>0.05).

When the level of beta-actin was set at 1.00, the levels of caspase-3 in the parathyroid cells from rabbits in the Sham group, CG, and CSG at 24 h after the treatments were 0.39±0.06, 0.84±0.12, and 1.12±0.12, respectively. The levels of caspase-3 in the parathyroid cells from rabbits in the Sham group, CG, and CSG at 72 h after the treatments were 0.39±0.05, 1.23±0.10, and 1.41±0.11, respectively. The levels of caspase-3 in the parathyroid cells from rabbits in the Sham group, CG, and CSG at 168 h after the treatments were 0.36±0.45, 0.65±0.12, and 0.44±0.10, respectively (Figure 4C). The results showed that the expression of caspase-3 in the parathyroid cells decreased significantly at 168 h after Ca\textsuperscript{2+} supplementation (P<0.05).

Discussion

In this study, a rabbit model of parathyroid gland ischemic injury was established. The results showed that Ca\textsuperscript{2+} inhibited the secretion of PTH and the apoptosis of parathyroid cells from 24–72 h after the Ca\textsuperscript{2+} supplementation, but the inhibition was not notable at 168 h after the Ca\textsuperscript{2+} supplementation. The serum free calcium levels in rabbits from the Sham group, CG, and CSG showed no significant difference before and after the specific treatments. The Western blotting analysis showed that Ca\textsuperscript{2+} supplementation inhibited the expression of p38 MAPK and caspase-3.

PTH, calcitomin, and 25-(OH)\textsubscript{2}D\textsubscript{3} (the active form of vitamin D) are involved in the regulation of calcium homeostasis [17–20]. The main role of PTH is to maintain adequate serum calcium levels by controlling bone calcium absorption [2,21], enhancing renal tubular calcium absorption [22], and stimulating active absorption of calcium in the intestine [21,23]. The main cells store relatively small amounts of hormones; they can respond to even mild stimuli caused by calcium in the serum. Serum calcium levels can be rapidly regulated by regulating the rate of hormone secretion and degradation [24,25]. Hypocalcemia leads to rapid release of PTH into the blood [26]; it has a rapid and direct effect, and when the plasma levels of calcium increase, the secretion of PTH is inhibited (negative feedback inhibition).

In this study, we established a rabbit model of unilateral parathyroid ischemic injury and treated it by Ca\textsuperscript{2+} supplementation; the serum free calcium levels in rabbits from the Sham group, CG, and CSG were the same before and after the treatments. Thus, it can be concluded that the sham operation did not affect the serum calcium level. In the CG, the serum calcium level may have been regulated by self-compensation after the ischemic injury of the parathyroid vessels. The elevation of PTH maintained the serum calcium concentration at the level observed before the ischemic injury; this was consistent with the finding reported by Bas [27,28], who established a rabbit model of acute hypocalcemia via intravenous EDTA infusion. It has been proposed that hypocalcemia can elevate the secretion of PTH from the basal to the maximal level. It is known that Ca\textsuperscript{2+} supplementation therapy for possible parathyroid injury can ameliorate hypocalcemia [16]; however, in this study, Ca\textsuperscript{2+} supplementation may have compensated for the hypocalcemia caused by the parathyroid injury due to calcium ingestion, at which time PTH maintains the serum calcium concentration to the same level as that observed before the operation. The PTH levels in rabbits from the CG at 24 h and 72 h after the treatment were higher than those at 24 h and 72 h before the operation, respectively; further, the apoptotic rate of parathyroid cells of rabbits from the CG was higher than that of those from rabbits in the CSG at 24 h and 72 h time. There was no difference between the PTH levels before and after the treatment in rabbits from the CG at 168 h, and there was no difference between the apoptotic rate of parathyroid cells of rabbits from the CG and CSG. This may be due to the damage of parathyroid function within 24 h after the injury of parathyroid vessels, which decreased the PTH level. The calcium level decreases, which promotes further PTH secretion by the parathyroid glands; hence, the PTH level in rabbits from the CG was higher at 24 h after the treatment than at 24 h before the treatment, and this process lasted until 72 h. During this period, the parathyroid glands in rabbits from the CSG were in a state of functional inhibition and did not need to produce additional PTH; this accelerates the repair process of the damaged parathyroid glands. At 168 h, due to parathyroid glands damage, the repair process is accelerated. Because the gland had begun to recover, the PTH level did not fluctuate significantly compared to before treatment, and at this time point, the apoptotic rate of cells of rabbits from the CG and CSG showed no difference.

Apoptosis is the main pathological change associated with parathyroid ischemic injury, accompanied by fibrosis and infiltration of inflammatory cells. Cell necrosis is not obvious, regardless of Ca\textsuperscript{2+} supplementation.

Normal parathyroid glands express many genes [29–32], but not their proteins [33]; they express the corresponding proteins only during pathological changes [34,35]. The apoptotic process plays an important role, especially in the maintenance of the structure of various glands [36,37]. In the model of parathyroid vascular injury, the expression of p38 MAPK and caspase-3 in rabbits from the CSG decreased. Because p38 MAPKs and caspase-3 are involved in the apoptotic process of cells in the damaged parathyroid gland, this finding indicates that Ca\textsuperscript{2+} supplementation can inhibit the apoptosis...
in these cells. The apoptosis-inhibiting effects of Ca\textsuperscript{2+} supplementation may be due to the mild inhibition of parathyroid gland activity after Ca\textsuperscript{2+} supplementation, which is conducive to functional recovery and tissue remodeling of the parathyroid gland. However, the specific molecular mechanisms underlying these phenomena and the specific roles of p38 MAPK in these processes need to be further studied.

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Conclusions

This study demonstrates that apoptosis can be a part of the pathological change associated with parathyroid ischemic injury, and Ca\textsuperscript{2+} can play an important role in inhibiting the apoptosis of parathyroid cells following ischemic injury.