Effect of Qingyitang on activity of intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in rats with acute pancreatitis

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AIM: To study the change of intracellular calcium-magnesium ATPase (Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase) activity in pancreas, liver and kidney tissues of rats with acute pancreatitis (AP), and to investigate the effects of Qingyitang (QYT) (Decoration for clearing the pancreas) and tetrandrine (Tet) and vitamin E (VitE) on the activity of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase.

METHODS: One hundred and five Sprague-Dawley rats were randomly divided into: normal control group, AP group, treatment group with QYT (1 ml/100 g) or Tet (0.4 ml/100 g) or VitE (100 mg/kg). AP model was prepared by a retrograde injection of sodium taurocholate into the pancreatic duct. Tissues of pancreas, liver, and kidney of the animals were taken at 1 h, 5 h, 10 h respectively after AP induction, and the activity of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase was studied using enzyme-histochemistry staining. Meanwhile, the expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase of the tissues was studied by RT-PCR.

RESULTS: The results showed that the positive rate of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in AP group (8.3%, 25%, 29.2%) was lower than that in normal control group (100%) in all tissues (P<0.01), the positive rate of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in treatment group with QYT (58.3%, 83.3%, 83.3%), Tet (70.0%, 75.0%, 75.0%) and VitE (54.2%, 75.0%, 79.2%) was higher than that in AP group (8.3%, 25.0%, 29.2%) in all tissues (P<0.01). RT-PCR results demonstrated that in treatment groups Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase gene expression in pancreas tissue was higher than that in AP group at the observing time points, and the expression at 5 h was higher than that at 1 h. The expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in liver tissue was positive, but without significant difference between different groups.

CONCLUSION: The activity and expression of intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase decreased in rats with AP, suggesting that Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase may contribute to the occurrence and development of cellular calcium overload in AP. QYT, Tet and VitE can increase the activity and expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and may relieve intracellular calcium overload to protect the tissue and cells from injuries.
with Tet (0.4 ml/100 g), and in AP+VitE group, rats were given VitE (100 mg/kg) intravenously through mesenteric vein.

**HE staining**
At 1 h, 5 h or 10 h after operation, the tissue samples of pancreas, liver and kidney were taken and fixed with formalin solution. Some sections of the specimens were stained with HE, and then observed under a light microscope.

**Enzyme histochemistry**
Some of the tissue blocks were used for enzyme histochemistry staining. Four μm thick tissue slices were mounted to polysilane-coated slides with a cryotome. Wachstein-Meis and lead nitrate method[5] was used to conduct enzyme histochemistry stain for intracellular Ca²⁺-Mg²⁺-ATPase. The slides were examined under a light microscope.

**RT-PCR**
Primers for Ca²⁺-Mg²⁺-ATPase gene were designed based on mRNA sequence of intracellular Ca²⁺-Mg²⁺-ATPase[6]. GAPDH gene was used as internal housekeeping gene. Total RNA of AP+NS group was 20 μg and RNA integrity was confirmed by agar gel electrophoresis. The reverse transcription system was at 94°C for 1 min, at 57°C for 1 min, followed by 35 cycles at 94°C for 5 minutes, and at 72°C for 1 min with more than 50% of stained cells were positive (++). Before reverse transcription, RNA integrity was confirmed by agar gel electrophoresis. The reverse transcription reaction was performed in a thermal cycler (PE480). PCR reaction solution contained 1 μl cDNA, 2 μl dNTP (2 mmol/L), 1 μl primers, 2.5 μl 10×buffer, 2U Taq polymerase, 18 μl water. PCR process was set at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 min, at 57°C for 1 min, and at 72°C for 1 min. Final extension was at 72°C for 10 minutes. The PCR products were examined on 1% agar gel electrophoresis. The bands were observed and photographed under Ultraviolet light.

**Statistical analysis**
Data were analyzed by χ² test and P<0.01 was considered significant.

**RESULTS**

**Pathological findings in pancreas, liver and kidney tissues of AP rats**

**Pancreas**
Large areas of hemorrhage were found in pancreatic tissue of AP+NS group. Acinar structure was obscure. There were large areas of necrosis. Some nuclei were lysed and disappeared. Apparently saponified spots were seen. A number of inflammatory cell infiltrations were observed in the peri-necrotic tissues. In AP+QYT, AP+Tet or AP+VitE groups, the pancreas only slightly swelled with sporadic bleeding and necrosis, and mild inflammatory infiltration in the pancreatic tissue.

**Liver**
In AP+NS group, degeneration and necrosis of the liver cells were found, and hepatocytes were disordered and some hepatic cords disappeared. In AP+QYT, AP+Tet or AP+VitE groups, hepatocytes were only degenerated and swelled with slight focal hemorrhagic necrosis.

**Kidney**
In AP+NS group, epithelial cells in the proximal convoluted renal tubule were observed with degeneration and necrosis. Hyperemia, swelling and inflammatory cell infiltration were seen in renal glomeruli. Only edema was found in proximal convoluted renal tubular epithelial cells in AP+QYT, AP+Tet and AP+VitE groups (Figure 1).

**Activity of intracellular Ca²⁺-Mg²⁺-ATPase in pancreas, liver and kidney tissues of AP rats**
Positive staines were diffusely distributed in cellular membrane and cytoplasm of pancreatic acinar cells, hepatocytes, and proximal renal tubule epithelial cells. Positive rate of Ca²⁺-Mg²⁺-ATPase stain in tissues of normal group was significantly higher than that of other groups. The lowest positive rate was found in AP+NS group (P<0.01). There was no significant difference between groups of AP+QYT, AP+Tet, and AP+VitE (P>0.05) (Table 1, Figure 2).

**Expression of Ca²⁺-Mg²⁺-ATPase in pancreas and liver tissues of AP rats**
By RT-PCR technique, the expression of Ca²⁺-Mg²⁺-ATPase in pancreas and liver of all groups was measured respectively. The gene fragment of Ca²⁺-Mg²⁺-ATPase was 451 bp. The amplified fragment of internal housekeeping gene GAPDH was 450 bp. The results showed that the highest expression of Ca²⁺-Mg²⁺-ATPase was in normal group, the lowest was in AP+NS group, and moderate in AP+QYT, AP+Tet and AP+VitE groups. The expression decreased with time in AP+NS group. While in AP+QYT, AP+Tet and AP+VitE groups, the expression increased with time. The expression of Ca²⁺-Mg²⁺-ATPase in liver had no significant difference between groups (Figure 3).

**Table 1** Positive rate of activity of intracellular Ca²⁺-Mg²⁺-ATPase in tissues of AP rats

| Group    | n  | Pancreatic acinar cells | Hepatocytes | Renal epithelial cells |
|----------|----|-------------------------|-------------|------------------------|
|          |    | - | + | +++ | ++++ | Positive (%) | - | + | +++ | ++++ | Positive (%) | - | + | +++ | ++++ | Positive (%) |
| Normal group | 9  | 0 | 5 | 4 | 100⁺ | 0 | 3 | 6 | 100⁺ | 0 | 5 | 6 | 100⁺ |
| AP+NS     | 24 | 22 | 0 | 8.3 | 18 | 5 | 1 | 25.0 | 17 | 5 | 2 | 29.2 |
| AP+QYT    | 24 | 10 | 7 | 7 | 58.3⁺ | 4 | 11 | 9 | 83.3⁺ | 4 | 12 | 8 | 83.3⁺ |
| AP+Tet    | 24 | 12 | 9 | 3 | 50.0⁺ | 7 | 9 | 8 | 70.8⁺ | 6 | 10 | 8 | 75.0⁺ |
| AP+VitE   | 24 | 11 | 7 | 6 | 54.2⁺ | 6 | 11 | 7 | 75.0⁺ | 5 | 11 | 8 | 79.2⁺ |

*P<0.01 vs AP+NS.*
Figure 1. Histopathological findings in AP rats before and after treatment, HE stains. A: Morphological changes in pancreatic tissue of AP rats before treatment, ×200. B: Morphological changes in pancreatic tissue of AP rats after treatment with QYT, ×200. C: Morphological changes in hepatic tissue of AP rats before treatment, ×200. D: Morphological changes in hepatic tissue of AP rats after treatment with VitE, ×200. E: Morphological changes in renal tissue of AP rats before treatment, ×400. F: Morphological changes in hepatic tissue of AP rats after treatment with Tet, ×400.

Figure 2. Enzyme histochemistry staining for intracellular Ca²⁺-Mg²⁺-ATPase in AP rats before and after treatment. A: Pancreatic tissue in AP rats before treatment, ×400. B: Pancreatic tissue in AP rats after treatment with QYT, ×400. C: Hepatic tissue in AP rats before treatment, ×200. D: Hepatic tissue in AP rats after treatment with VitE, ×200. E: Renal tissue in AP rats before treatment, ×400. F: Renal tissue in AP rats after treatment with Tet, ×400.

Figure 3. Expression of Ca²⁺-Mg²⁺-ATPase in AP rats before and after treatment analyzed by RT-PCR. A: Expression of Ca²⁺-Mg²⁺-ATPase mRNA in pancreatic tissue of different groups. B: Expression of Ca²⁺-Mg²⁺-ATPase mRNA in hepatic tissue of different groups. C: amplification product of GAPDH gene in pancreatic tissue of different groups. D: amplification product of GAPDH gene in hepatic tissue of different groups. Note: 1: AP 1 h, 2: AP 5 h, 3: QYT+AP 1 h, 4: QYT+AP 5 h, 5: Normal control group, 6: Tet+AP 1 h, 7: Tet+AP 5 h, 8: VitE+AP 1 h, 9: VitE+AP 5 h, M: PCR marker.
DISCUSSION
The level of intracellular free calcium (Ca\(^{2+}\)) is not only dependent on the inflow of extracellular calcium through cell membrane and release from calcium reservoir inside the cell, but also on the function of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase on cell membrane and membrane of endoplasmic reticulum and mitochondria. By Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase, Ca\(^{2+}\) could be pumped out of cell or into calcium reservoir. Thus Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase could play an important role in intracellular calcium homeostasis[19].

Activity and expression of intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in AP and its implication
Our experimental results showed that, in AP rats, the activity of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in pancreatic, hepatic and renal tissues was decreased in AP rats. At the same time the pathological findings were aggravated. These results suggested that alteration of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity in AP might take part in the occurrence and progression of AP. It was reported that permeability of cell membrane was increased in AP, Ca\(^{2+}\) inflow might increase and lead to intracellular calcium overload. In the meantime, some stimulating factors could activate corresponding receptors on the surface of membrane to activate guanylate cyclation (GC). As a result, energy was released to cascade effector phospholipase C intracellular phosphatidylinositol diphosphate (PIP\(_2\)) and diacylglycerol (DG). The IP\(_3\)-Mg\(^{2+}\)-ATPase activity decreased in AP rats. At the same time the pathological findings were aggravated. These results suggested that intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity could be affected by the expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in AP rats, which suggested that intracellular calcium overload further facilitated the release of pro-inflammatory mediators, which would cause strong contraction and thrombosis of microcirculation. Thus energy metabolism in tissues was disordered and ATP production was reduced[12-18]. In addition, large quantities of free radicals produced during acute pancreatitis would cause phospholipids re-distribution in cell membrane. All of these factors might contribute to the inhibition of ATPase activity, which in turn would aggravate intracellular calcium overload. This vicious cycle occurred. Therefore, decrease of intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity plays a key role in the development and aggravation of calcium overload.

In the present study, we found that activity of intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase was decreased in hepatocyte of AP rat, but the expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in the tissues did not change greatly. This finding suggested that intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity was not only dependent on the level of its gene expression but also was affected by many other factors[17-19].

Therapeutic effect and mechanism of QYT, Tet and VitE
Chinese medicine QYT is an effective compound in the treatment of AP. It has been proved to have bacteriostatic and anti-inflammatory effects, and to promote intestinal movement[20,21]. Tet is a kind of bisbenzylisoquinoline alkaloid extracted from root tuber of Stephania tetrandra, a Chinese herbal medicine. It has been proved to be a natural non-selective calcium channel blocker[22-23], and VitE has also been proved to be a scavenging agent of free radicals and blocker for lipid peroxidation[24-27]. The present study found that in AP rats, intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity in pancreatic, hepatic and renal tissues was increased after treatment with the above three medicines, and in pancreas the expression of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase was enhanced. Furthermore, pathological changes of hemorrhage and necrosis in the tissues were relieved. The complicating ascites and pleural effusion were improved[28-34].

In summary, QYT, Tet and VitE have certain protecting effects on tissues and cells in AP, and the mechanisms are related with improved blood supply, increased intracellular Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity and reduced intracellular calcium overload.

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