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• BASIC RESEARCH •

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

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Abstract

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) (Decoction 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 (50.0%, 70.8%, 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.

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INTRODUCTION

The pathogenesis of acute pancreatitis (AP) is complicated. A number of theories have been proposed. It is generally accepted that "calcium overload" plays a key role in the occurrence and progression of AP[1-3]. However, the exact mechanism of intracellular calcium overload in acute pancreatitis is not clear yet. This study was designed to explore the mechanism of intracellular calcium overload by determining the activity of intracellular Ca^{2+}-Mg^{2+} ATPase in AP rats. The experiment also investigated the therapeutic mechanisms of some medicines, such as Chinese medicines Qingyitang (QYT) and tetrandrine (Tet), and Vitamin E (VitE) on AP in rats.

MATERIALS AND METHODS

Animals

One hundred and five Sprague-Dowley (SD) rats including male and female (1:1) were used. The animals, weighing 220-250 g, were provided by the Animal Center of Chinese Academy of Sciences, Shanghai, China.

Reagents

Sodium taurocholate from Sigma Co. was diluted with distilled water to make a 4% solution for use. QYT was from Zunyi Medical College, Tet from the Pharmaceutical Institute of the Second Military Medical University, VitE (emulsion, 2 ml/kg) from Xinan Pharmaceutical Factory, ATP disodium salt from Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Trizol, reverse transcriptase Superscript II RNase H- were from Gib Co. (USA), hexamer from Promega (USA), and Taq DNA polymerase, dNTP, RNAase inhibitor from Takara Co. (Japan), diethyl pyrocarbonate (DEPC) from Serva Co. (USA). Primers for Ca^{2+}-Mg^{2+}-ATPase (upstream primer -5' GAACATCCTGCAGACGGACA-3', downstream primer -5' CAAAGCTATGGGAGTGGTGG-3') (790-1 241 bp) and primers for GAPDH (upper - 5' ACCACAGTCCATGCCAT CAC-3', lower - 5' TCCACCACCCTGTTGCTGTA-3') were purchased from Takara Co. (Japan).

Animal model preparation and grouping

The rats were randomly divided into normal control group (n=9), AP+ normal saline (NS) group (n=24), AP+QYT group(n=24), AP+Tet group (n=24), AP+VitE group (n=24). In normal control group the pancreas of rats were exposed. In the other groups, the pancreas of rats was exposed and sodium taurocholate was injected into the pancreatic duct to induce AP[4]. In AP+NS group, rats were injected with NS (0.4 ml/100 g, ip) after AP induction. In AP+QYT group, rats were infused with QYT (1 ml/100 g) by a nose-gastric catheter, in AP+Tet group, rats were injected peritoneally

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 polylysine-coated slides with a cryotome. Wachstein-Meisd lead nitrate method[5] was used to conduct enzyme histochemistry stain for intracellular Ca^{2+}-Mg^{2+}-ATPase. The slides were examined under a light microscope.

RT-PCR

Primers for Ca^{2+}-Mg^{2+}-ATPase gene were designed based on mRNA sequence of intracellular Ca^{2+}-Mg^{2+}-ATPase[6]. GAPDH gene was used as internal housekeeping gene. Total RNA of pancreas and liver were taken for RT-PCR at 1 h and 5 h after AP was induced. Total RNA was extracted with Trizol reagents. Quantity and purity of RNA were determined with an ultraviolet spectrophotometer. RNA integrity was confirmed by agar gel electrophoresis. The reverse transcription system was 20 μl in volume, containg 2 μg total RNA, 1 μl reverse transcriptase, 1 μl dNTP (10 mmol/L), 2 μl (100 ng/1 μl) hexamer, 4 μl 5×buffer, 2 μl 0.1M DTT, 8 μl water. The mixture was incubated at 37 ℃ for 1 hour and then at 70 ℃ for 15 minutes to inactivate reverse transcriptase. cDNA was used as a template for subsequent PCR. PCR 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 at 94 ℃ for 5 minutes, followed by 35 cycles at 94 ℃ for 1 min, at 57 ℃ for 1 min, and at 72 ℃ for 1 min. Final extension was at 72 ℃ for 10 minutes, 8 μl of PCR products was examined on 1% agar gel electrophoresis. The bands were observed and photographed under Ultraviolet light.

Statistical analysis

Data were analyzed by χ^2 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 Ca^{2+}-Mg^{2+}-ATPase in pancreas, liver and kidney tissues of AP rats

Positive stains were diffusely distributed in cellular membrane and cytoplasm of pancreatic acinar cells, hepatocytes, and proximal renal tubule epithelial cells. Positive rate of Ca^{2+}-Mg^{2+}-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).

Positive cells were defined as cells with uneven chocolate brown particles in cytoplasm under light microscope. The darker the staining color was, the higher the activity was. The cells were classified into 4 levels based on color intensity and number of positive cells. No staining or only a small number of light brown particles in cellular membrane and cytoplasm, and more than 25% of stained cells were negative (-); with a medium number of brown particles, and 25%-50% of stained cells were positive (+); with a large number of brown particles and more than 50% of stained cells were positive (++); more than 75% of stained cells were positive (+++).

Expression of Ca^{2+}-Mg^{2+}-ATPase in pancreas and liver tissues of AP rats

By RT-PCR technique, the expression of Ca^{2+}-Mg^{2+}-ATPase in pancreas and liver of all groups was measured respectively. The gene fragment of Ca^{2+}-Mg^{2+}-ATPase was 451 bp. The amplified fragment of internal housekeeping gene GAPDH was 450 bp. The results showed that the highest expression of Ca^{2+}-Mg^{2+}-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 Ca^{2+}-Mg^{2+}-ATPase in liver had no significant difference between groups (Figure 3).

Table 1 Positive rate of activity of intracellular Ca^{2+}-Mg^{2+}-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[a]	0	3	6	100[a]	0	5	6	100[a]
AP+NS	24	22	2	0	8.3	18	5	1	25.0	17	5	2	29.2
AP+QYT	24	10	7	7	58.3[a]	4	11	9	83.3[a]	4	12	8	83.3[a]
AP+Tet	24	12	9	3	50.0[a]	7	9	8	70.8[a]	6	10	8	75.0[a]
AP+VitE	24	11	7	6	54.2[a]	6	11	7	75.0[a]	5	11	8	79.2[a]

[a]$P<0.01$ *vs* AP+NS.

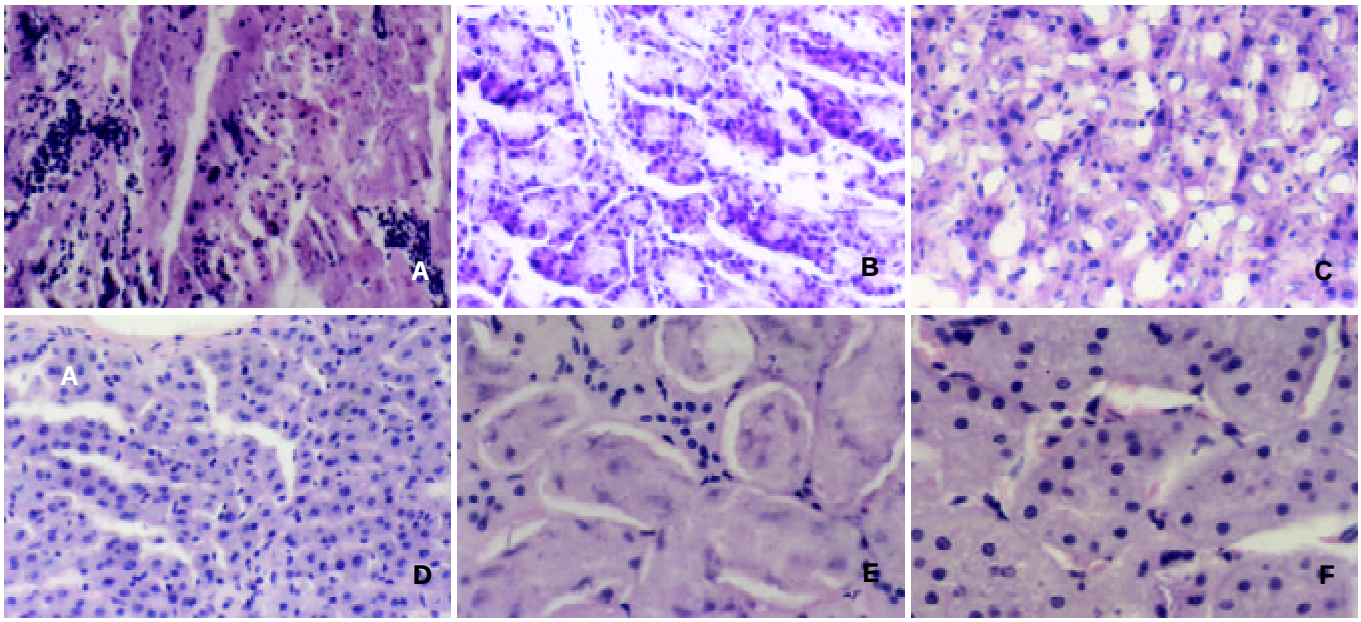

Figure 1 Histopathological findings in AP rats before and after treatment, HE stains. A: Morphological changes in pancreatic tissue of AP rats before treatment, ×200. B: Morphological changes in pancreatic tissue of AP rats after treatment with QYT, ×200. C: Morphological changes in hepatic tissue of 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.

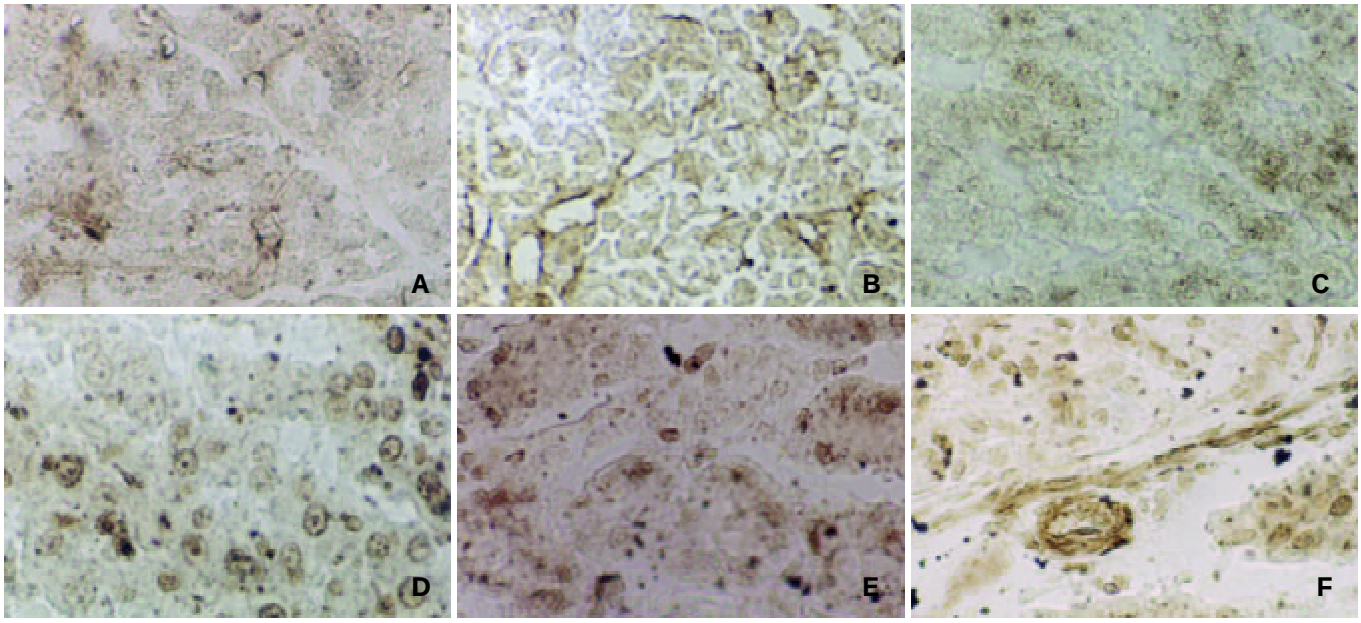

Figure 2 Enzyme histochemistry staining for intracellular Ca^{2+}-Mg^{2+}-ATPase in AP rats before and after treatment. A: Pancreatic tissue in AP rats before treatment, ×400. B: Pancreatic tissue in AP rats after treatment with QYT, ×400. C: Hepatic tissue in AP rats before treatment, ×200. D: Hepatic 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.

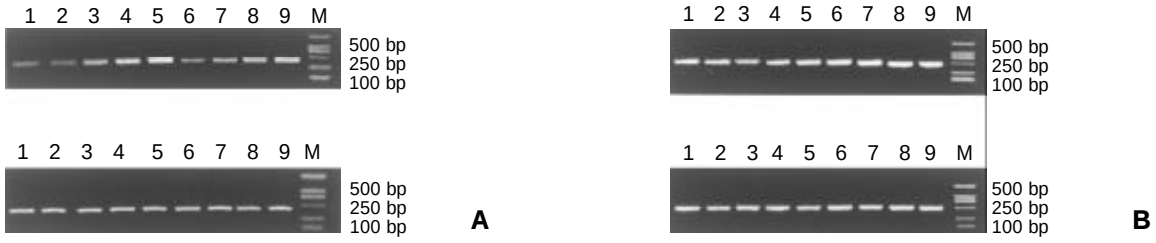

Figure 3 Expression of Ca^{2+} -Mg^{2+} -ATPase in AP rats before and after treatment analyzed by RT-PCR. A: Expression of Ca^{2+} -Mg^{2+} -ATPase mRNA in pancreatic tissue of different groups. B: Expression of Ca^{2+} -Mg^{2+} -ATPase mRNA in hepatic tissue of different groups. C: amplification product of GAPDH gene in pancreatic tissue of different groups. D: amplification product of GAPDH geneinhepatic 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+AP1 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[7-9].

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) into inositol triphosphate (IP_3) and diacylglycerol (DG). The IP_3 subsequently activated IP_3 receptors on endoplasmic reticulum to stimulate Ca^{2+} release from calcium reservoir. Consequently intracellular Ca^{2+} level increased abruptly. If the intracellular Ca^{2+}-Mg^{2+}-ATPase activity was decreased, Ca^{2+} could not be effectively pumped back into the reservoir or out of cells, which further aggravated intracellular calcium overload[10,11]. 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[12-16]. 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. The 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 tetrandran, 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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