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Protective effect of low-molecular-weight heparin on pancreatic encephalopathy in severe acute pancreatic rats

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Abstract
Background and aims Pancreatic encephalopathy (PE) is a severe complication and significant cause of death in patients with severe acute pancreatitis (SAP). We have reported previously that low-molecular-weight heparin (LMWH) treatment could reduce incidence of PE in SAP patients. Our objective here was to investigate the protective effect of LMWH and its mechanism on PE in SAP rats.
Methods SD rats were randomly divided into three groups: (1) Sham-operation (S) group, (2) SAP group, and (3) LMWH treatment (LMWH) group. LMWH was administrated 4 h after the SAP model conducted. The levels of serum amylase, myelin basic protein (MBP), tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), brain water content, occurrence of apoptosis, and pathological changes of pancreas and brain were measured at 1 day after models were set up in the SAP and S groups, and 1 day after LMWH treatment was administrated in the LMWH group.
Results (1) The levels of serum amylase, TNF-α, and IL-6 in the SAP group were increased significantly more than those in the S and LMWH groups (all $P < 0.001$), as were the levels of serum MBP in the SAP group compared to those in the S and LMWH groups ($P < 0.01$, <0.05 respectively). However, while the level of serum amylase and IL-6 in the LMWH group were significantly increased compared to those in the S group ($P < 0.05$, <0.001 respectively), the levels of TNF-α and MBP showed no significant difference between the LMWH and S groups (all $P > 0.05$). (2) The brain water content in the SAP group was significantly increased compared to the S group and LMWH group ($P < 0.01$, <0.05 respectively). (3) Neuronal apoptosis, demyelination, and mitochondrial vacuolation in neuronal cells were observed in the SAP group; in contrast, in the LMWH group, significantly lower rates of neuronal apoptosis, demyelination and mitochondrial edema were observed in neuronal cells.
Conclusions The protective effect of LMWH on PE progression in SAP rats might result from inhibition of inflammatory activation and reduction of the occurrence of neuronal apoptosis.

Keywords Low-molecular-weight heparin · Severe acute pancreatitis · Pancreatic encephalopathy

Abbreviation

LMWH	Low-molecular-weight heparin
SAP	Severe acute pancreatitis
PE	Pancreatic encephalopathy
LT	Low-molecular-weight heparin treatment
S	Sham operation
ELISA	Enzyme-linked immunosorbent assay
MBP	Myelin basic protein
TNF-α	Tumor necrosis factor-alpha
IL-6	Interleukin-6
TNM	Tumor node metastasis

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Introduction

SAP is a systemic disease usually complicated by systemic inflammatory reaction syndrome and multiple organ dysfunctions; SAP patients mainly die of organ failure. PE is a severe complication and an important cause of death in SAP patients; its mortality rate can reach up to 67–100 % [1]. Therefore, reducing occurrence of PE is very important for decreasing the mortality rate in SAP patients. LMWH decreases endotoxin 1, leading to improvement of microcirculation system function, and has anti-thrombus effects to reduce the formation of microthrombosis in the brain. We have recently shown that LMWH has an important effect on the prevention of PE and clinical treatment of SAP, which is related to the reduction of microthrombosis and improvement of microcirculation in pancreas and brain tissues [2–4].

In the present study, we investigated the protective effect of LMWH on PE progression in SAP rats. By morphological examination and ELISA assays, the serum levels of amylase, myelin basic protein (MBP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), brain water content, and pathological changes of the brain were detected in the SAP group, the sham-operation (S) group, and the LMWH treatment (LT) group. The experimental results demonstrate that LT can significantly reduce the occurrence of PE in SAP rats.

Materials and methods

Materials

Sodium taurocholate was purchased from Sigma Co., USA. LMWH was purchased from Hangzhou Jiuyuan Gene Engineering Co., Ltd, China. TNF-α, IL-6, and MBP ELISA kits were, respectively, bought from Beijing Jingmei Biotech Co., Ltd, China; Shanghai Senxiong Science and Technology Industrial Co., Ltd, China; and the Institute of Biochemistry and Molecular Biology, Chengdu Huaxi Medical University, China.

Animal studies

Sixty-four healthy Sprague–Dawley rats (weight 250–300 g, from the Experimental Animals Center of the Xiangya School of Medicine, Central South University, China) were randomly divided into three groups: (1) the S group, in which 16 rats underwent laparotomy only, without SAP induction or LMWH administration; (2) the SAP group ($n = 24$), in which SAP was induced by intraductal injection of 5 % sodium taurocholate into the pancreatic duct, but LMWH was not administered; (3) the LMWH group ($n = 24$), in which LMWH (100 IU/kg, Q 12 h, administered twice) was subcutaneously injected 4 h after SAP induction. All procedures were conducted according to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

Model construction

The rats fasted for 24 h, and 3 ml/kg gentamycine (320,000 U/L) was subcutaneously injected, after which 3 % pentobarbital (30 mg/kg) was intraperitoneally injected for anesthesia. Laparotomy was performed in aseptic conditions. A catheter was inserted into the pancreatic duct with the outboard clamped, and retrograde intraductal injection (2 ml/min) of 5 % sodium taurocholate (1 ml/kg) into the pancreatic duct was performed. Two min after the injection, the clamp was removed. The portal venous blood specimens and tissues of brain were obtained 1 day after the models were set up in both the SAP and S groups and 1 day after LMWH administration in the LMWH group.

Determination of serum levels of amylase

TNF-α, IL-6 and MBP

Serum amylase was detected by a protein analyzer (HITACHI 7060 Automatic Analyzer, Japan). The serum levels of TNF-α, IL-6 and MBP were determined by ELISA according to the manufacturer's instructions, using commercially paired antibodies and recombinant standards obtained from each of the ELISA kits.

Brain water content

Brain water content was measured using the dry–wet weight method. Brain water content = (brain wet weight–brain dry weight)/brain wet weight.

Transmission electron microscopic examination of the brain

The morphology of the frontal cortical tissues of brain was examined 1 day after models were set up in the SAP and S groups and 1 day after LMWH administration in the LMWH group using a transmission electron microscope at the Xiangya School of Medicine Electron Microscope Facility, Central South University, China. The tissues were fixed in phosphate-buffered 2.5 % glutaraldehyde for 3 h, postfixed in phosphate-buffered 2 % osmium tetroxide for 1 h, dehydrated in ascending concentrations of acetone, and infiltrated over 24 h with Spurr's resin. Ultrathin sections (60 nm) of frontal cortical tissues were stained with

uranyl and lead salts and then observed using a Hitachi-600 transmission electron microscope (Japan).

Detection of apoptotic neurons

The terminal dUTP nick end-labeling (TUNEL) assay was performed using the TdT-FragEL TM DNA Fragmentation Detection kit (Calbiochem/Oncogene Research Products, Cambridge, USA) according to the manufacturer's instructions. Briefly, 4 μm sections from the paraffin-embedded samples were dewaxed with xylene and hydrated using graded alcohols, and the specimens were treated with 20 mg/ml proteinase K for 5 min and with 0.6 % H_2O_2 in methanol to eliminate endogenous peroxidase activity. Afterward, the sections were treated with the TDT enzyme and immersed in a biotinylated nucleotides solution. Apoptotic cells were detected using streptavidin–peroxidase conjugate followed by diaminobenzidine staining. The apoptotic index was determined from the total number of neuronal apoptosis in six high power fields (original magnification: 400×).

Statistical analysis

The data were presented as the mean ± standard deviation (SD). Statistical differences were compared by one-way analysis of variance (ANOVA) followed by pair-wise comparisons with Fisher's protected least significant difference (PLSD) procedure. $P < 0.05$ was considered statistically significant.

Results

Serum levels of amylase, TNF-α, IL-6, and MBP

By studying the effect of LT on SAP, we determined the ability of LMWH to inhibit secretion of amylase, TNF-α, IL-6, and MBP. The serum levels of amylase, TNF-α, and IL-6 in the SAP group were significantly higher than those in the S and LMWH groups (all $P < 0.001$). The serum level of MBP in the SAP group was significantly higher than that in the S and LMWH groups ($P < 0.01$, <0.05, respectively), and the serum levels of amylase and IL-6 in the LMWH group were significantly higher than those in the S group ($P < 0.05$, <0.001, respectively). However, the serum levels of TNF-α and MBP were not significantly different between the LMWH group and the S group (all $P > 0.05$, Table 1).

Brain water content

The brain water content in the SAP group (0.80 ± 0.46) was significantly higher than that of the S group (0.76 ± 0.06) and the LMWH group (0.76 ± 0.07) ($P < 0.01$, <0.05, respectively), whereas the brain water contents in the S group and the LMWH group were not significantly different ($P > 0.05$).

Morphological examinations of the brain

Under a transmission electron microscope, we observed nuclear condensation and margination of neuronal cells, endoplasmic reticulum expansion, neuronal apoptosis, demyelination of myelinated nerve fibers, and mitochondrial vacuolation of neuronal cells and glial cells in the frontal cortical tissues in the SAP group. However, in the LMWH group, significantly lower rates of neuronal apoptosis, demyelination and mitochondrial edema were observed in neuronal cells (Fig. 1). These results indicate that LMWH treatment can ameliorate the brain damage in SAP rats.

Apoptotic index

The apoptotic index in the SAP group was significantly higher than that in the S and LMWH groups (all $P < 0.0001$), and the apoptotic index in the LMWH group

Table 1 Serum amylase (U/l), TNF-α (pg/ml), IL-6 (pg/ml), and MBP (μg/l)

Group	*n*	Amylase	TNF-α	IL-6	MBP
S group	16	587.42 ± 15.08[a]	21.07 ± 3.94[a]	14.60 ± 4.16[a]	6.58 ± 0.29[d]
SAP group	24	8284.69 ± 346.01	60.02 ± 32.47	131.73 ± 56.33	9.32 ± 0.77
LMWH group	24	1650.61 ± 145.49[a,b]	30.89 ± 9.32[a]	21.57 ± 8.36[a,c]	7.51 ± 0.31[e]

[a] Compared with SAP group, $P < 0.001$

[b] Compared with S group, $P < 0.05$

[c] Compared with S group, $P < 0.001$

[d] Compared with SAP group, $P < 0.01$

[e] Compared with SAP group, $P < 0.05$

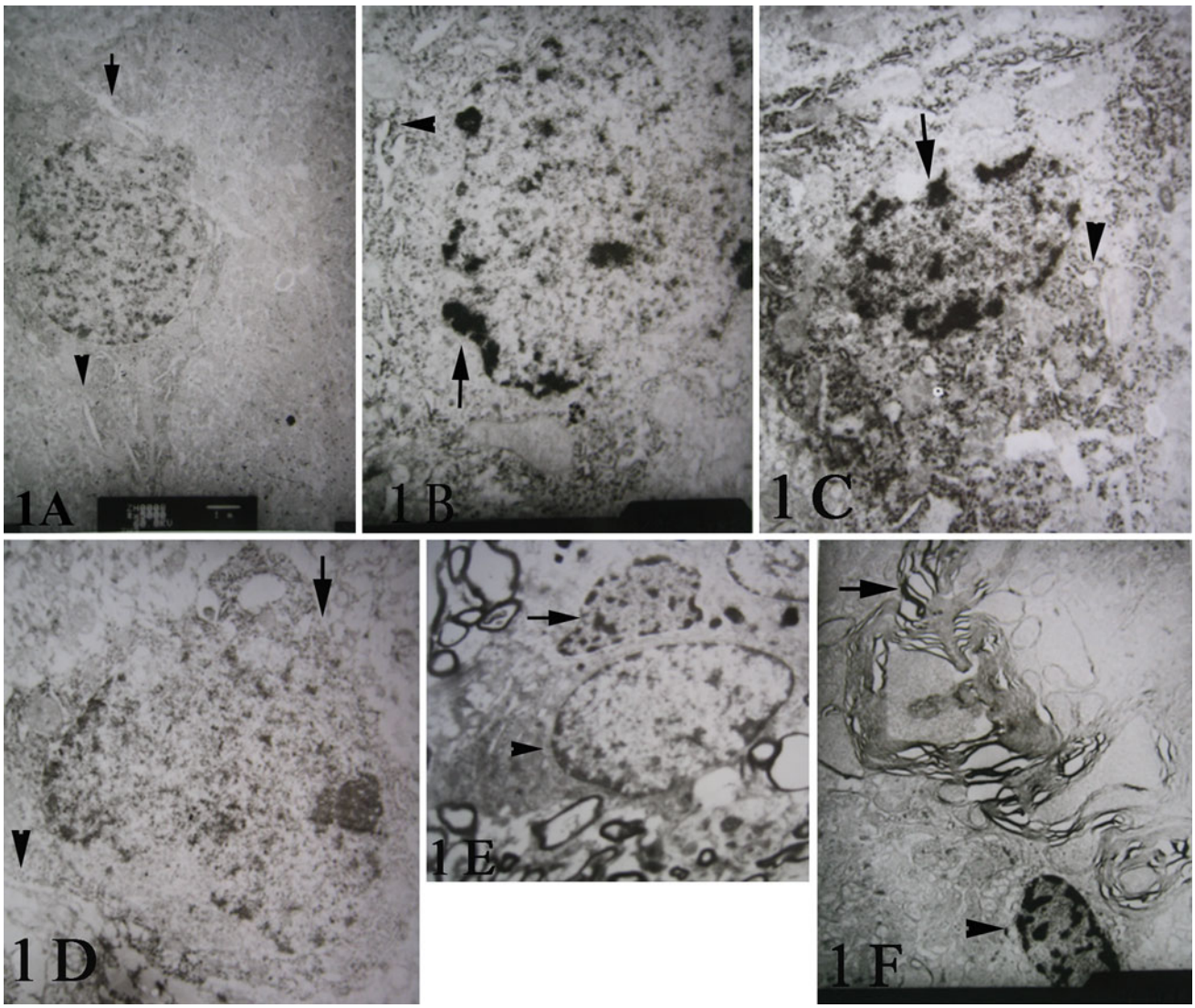

Fig. 1 Transmission electron microscopic examination of the rat brains were detected at 1 day after models were set up in the SAP and S groups, and 1 day after LMWH was administered in the LMWH group. **a** Normal neuronal cells (*arrow*), mitochondria, and endoplasmic reticulum (*arrowhead*) in the S group were detected at objective magnifications; ×5,000. **b** Nuclear condensation and margination of the neuronal cells (*arrow*) and expansion of the endoplasmic reticulum (*arrowhead*) of the SAP model were observed at objective magnifications; ×12,000. **c** Neuronal apoptosis (*arrow*) and mitochondrial vacuolation in neuronal cells (*arrowhead*) were indicated within neuronal cells in the SAP model at objective magnifications; ×8,000. **d** In the LMWH group, there were significantly fewer mitochondrial edema (*arrowhead*) of neuronal cells than those in the SAP group, while nearly normal neural structures (*arrow*) were seen at objective magnifications; ×8,000. **e** Normal myelinated nerve (*arrow*) and glial cells (*arrowhead*) in the brain tissues from an S rat were examined at objective magnifications; ×6,000. **f** Demyelination of myelinated nerve (*arrow*), and mitochondrial vacuolation in glial cells (*arrowhead*) of the SAP group were observed at objective magnifications; ×6,000

was significantly higher than those in the S group ($P < 0.001$) (Fig. 2). These results indicated that LMWH treatment can decrease neuronal apoptosis in SAP rats.

Discussion

The pathogenesis of PE is still not completely understood; it may involve many factors, such as an increase in blood–brain barrier permeability in SAP, allowing toxic factors to enter the brain venously. Therefor, SAP-induced factors including trypsin, elastase, phospholipase A2, vascular kallikrein, kinin, and proinflammatory cytokines (such as TNF-α and IL-6) can cause cerebral venous thrombosis, nerve cell swelling, central nervous system metabolic disorder, small intracerebral hemorrhage, and cerebral edema and softening, leading to neurological disorders [5–7].

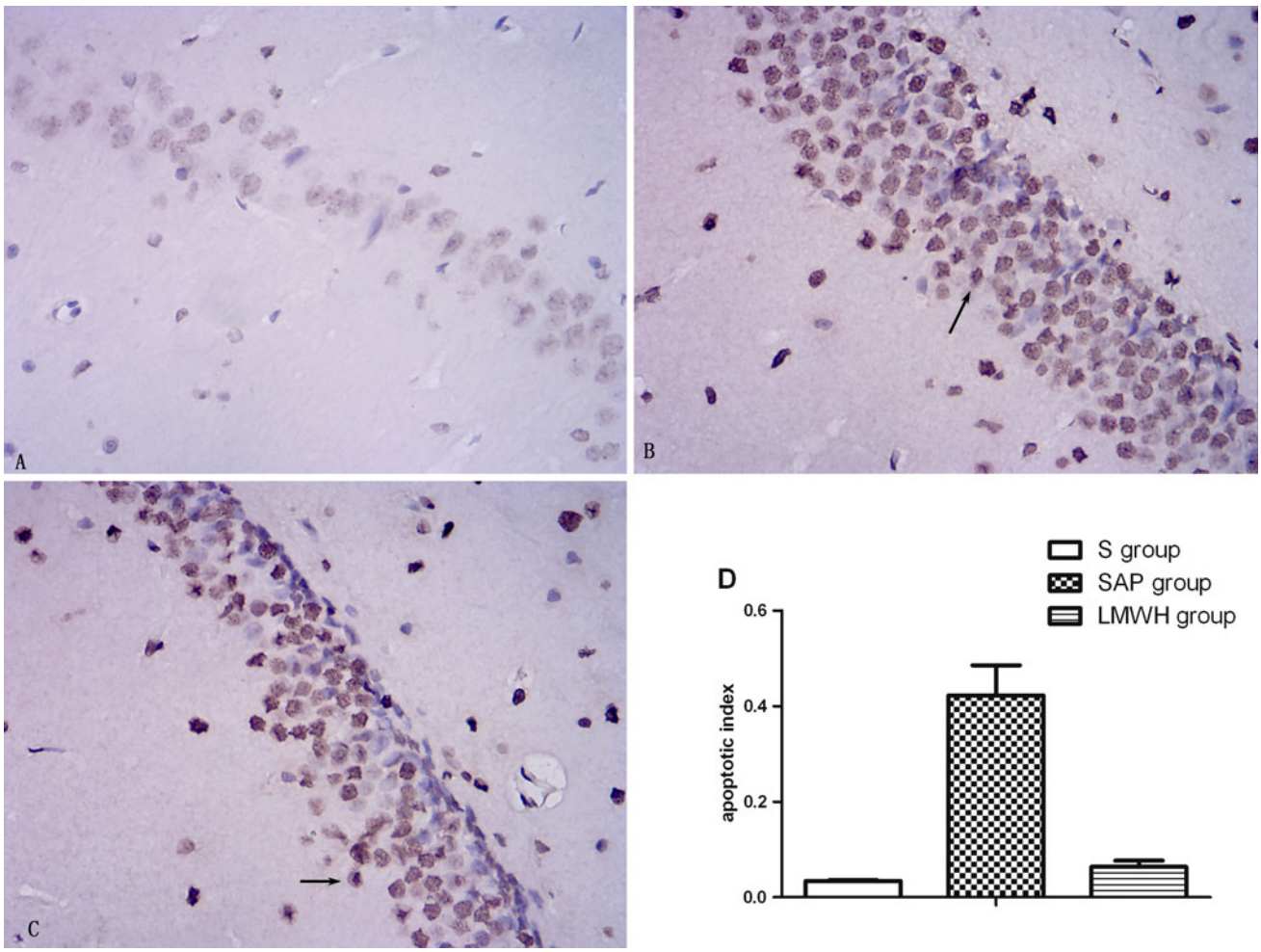

Fig. 2 TUNEL labeling of the rat brains was detected at 1 day after models were set up in the S (**a**) and in SAP group (**b**), and 1 day after LMWH was administered in the LMWH group (**c**). Apoptotic cells (*arrow*) can be seen (original magnification: 400×). The apoptotic index (**d**) in the SAP group was significantly higher than those in the S and LMWH groups (all $P < 0.0001$), and the apoptotic index in the LMWH group was significantly higher than that in the S group ($P < 0.001$). Data are shown as the *mean* ± *SEM* of the apoptotic index

Farkas et al. reported that the serum levels of TNF and IL-6 were increased in experimental pancreatitis, resulting in a significant increase in blood–brain barrier permeability. The serum TNF level was increased 6 and 24 h after the induction of pancreatitis, while the IL-6 level increased after 24 and 48 h. They hypothesized that proinflammatory cytokines (such as TNF and IL-6) may lead to vasogenic brain edema formation during acute pancreatitis [8]. In the present study, we observed that the serum level of amylase, TNF-α, and IL-6, as well as the brain water content, was significantly increased in the SAP group, and myelinated nerve fibers from the brain were demyelinated. Therefore, we considered that the elevated serum levels of amylase, TNF-α, and IL-6 induced by SAP might lead to brain damage. MBP is a protein isolated from myelin membranes and believed to be very important in the process of nerve myelination in the central nervous system [9]. The damage of the central nervous system and the increased permeability of the blood–brain barrier allow MBP to enter the circulation, leading to an increased level of serum MBP. As Strand et al. [10] reported, patients with cerebral infarction and hemorrhage had higher than normal MBP concentrations in cerebrospinal fluid, and the MBP level was also closely correlated to the visibility of cerebral lesions on a CT scan and to the short-term outcome of patients with cerebral infarction and hemorrhage. They concluded that brain-specific MBP is a useful marker for brain damage and severity of acute cerebrovascular diseases. Our data demonstrated that the serum levels of amylase and MBP, brain water content, and the severity of brain damage were significantly decreased in the LMWH group compared to those in the SAP group. These results suggest that LMWH treatment can inhibit the secretion of inflammatory cytokines, and ameliorate pancreatic damage and brain damage

in SAP. Our previous studies showed that the effects of LMWH treatment were significantly inhibited by the secretion of MBP, TNF-α and IL-6 in the patients with SAP (data not shown), and these results were in agreement with the results from the current experiment.

Recently, a growing body of evidence showed that the potential induction of pancreatic acinar cell apoptosis may be beneficial in the clinical management of acute pancreatitis, but reports of neuronal apoptosis in SAP are rare [11–13]. In this study, nuclear condensation and margination were observed in the SAP group, indicating early onset and progression of apoptosis within neuronal cells in SAP. We believe that this brain damage was due to neuronal apoptosis and proinflammatory cytokines secretion. Mitochondria are the initiation site of the intrinsic apoptotic cascade, and changes in the structure and permeability of mitochondrial membranes have been demonstrated to play important roles in the initiation and progression of apoptosis [14, 15]. Hao et al. reported that LMWH significantly increased neuronal cell viability and the protein expression levels of bcl-2, and decreased the LDH release, the number of apoptotic cells, the concentration of intracellular Ca(2+) and the protein expression levels of caspase-3 in cortical neurons, suggesting the potential mechanisms for SAP-induced neuronal apoptosis are protected by LMWH [16]. The brain tissues from the SAP group clearly showed that vacuolar degeneration of mitochondria was present in both the early and late apoptotic stages, and these results suggested that SAP-induced mitochondria degeneration might involve initiation and progression of neuronal apoptosis. In this study, the LMWH treatment was capable of inhibiting neuronal apoptosis and exerting a protective effect on mitochondria against SAP-induced mitochondria degeneration.

Talley et al. demonstrated a direct and proportional relationship between increasing numbers of neuronal apoptosis and increasing doses of TNF-α ranging from 0.05 to 25 ng/ml in a human neuroblastoma cell line, and they further proved that reactive oxidative intermediates were required for TNF-α-mediated neuronal apoptosis because neuronal apoptosis could be completely blocked by the antioxidant *N*-acetylcysteine [17]. The decrease of TNF-α after LMWH treatment might be able to block SAP-related neuronal apoptosis and increase neuronal survival. In this study, we found that SAP-induced brain damage was ameliorated by LMWH treatment. In addition, our data showed that the effects of LMWH treatment were significantly inhibited by the secretion of amylase, TNF-α, and IL-6 in the SAP group, suggesting that blocking the inflammatory reaction may inhibit the development of pancreatitis and brain damage, an effect which might play an important role in LMWH-treated SAP. Furthermore, after LMWH treatment, SAP-induced mitochondrial degeneration was also reduced and neuronal apoptosis disappeared, strongly indicating that SAP-induced neuronal apoptosis could be protected by LMWH. Taken together, we suggest that the protective mechanism of LWMH for SAP patients might result from the inhibition of inflammatory activation and reduction of the occurrence of neuronal apoptosis.

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