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# Effects of lipopolysaccharides on calcium homeostasis in isolated pancreatic acinar cells of rat[1]

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**KEY WORDS** lipopolysaccharides; pancreas; acinar cells; calcium; confocal microscopy

## ABSTRACT

**AIM:** To investigate the effects of lipopolysaccharides (LPS, endotoxin) on the calcium content in pancreatic acinar cells and the origin of $Ca^{2+}$ during calcium overload induced by LPS, further to explore the mechanism of LPS in inducing calcium overload and pancreatic acinar cell injury. **METHODS:** Male rat pancreatic acinar cells were isolated by collagenase digestion and loaded with Fluo-3/AM, then exposed to varying doses of LPS (from 1 mg/L to 20 mg/L). The dynamic change of $[Ca^{2+}]_i$ in single pancreatic acinar cell in the absence and presence of $Ca^{2+}$ in extracellular fluid was determined by laser scanning confocal microscopy. Cell viability was determined by MTT at different time points after treatment with LPS. **RESULTS:** Under physiological calcium concentration in extracellular fluid, LPS (10 mg/L) initiated a rapid, concentration-dependent rise in intracellular $[Ca^{2+}]_i$ and consequent cell damage ($P<0.05$). LPS induced a slight rise of $[Ca^{2+}]_i$ in the calcium-free extracellular fluid containing egtazic acid 1 mmol/L and addition of extracellular calcium in the presence of LPS resulted in a more immediate and remarkable rise of $[Ca^{2+}]_i$, which reached the peak value within 150 s and maintained the value sustainedly. Egtazic acid attenuated LPS-induced cell damage ($P<0.05$). The increase in intracellular $[Ca^{2+}]_i$ preceded the pathological alteration of pancreatic acinar cells. **CONCLUSION:** LPS directly induced the injury and the disorder of calcium homeostasis in isolated rat pancreatic acinar cell. Calcium overload is an early event in the pathogenesis of LPS-induced cell damage. Origin of the $[Ca^{2+}]_i$ in cytoplasma of pancreatic acinar cells during calcium overload is mainly due to the influx of extracellular $Ca^{2+}$. Calcium homeostasis disorder may be one of the causes or at least an important mediator of LPS-induced pancreatic acinar cell damage.

## INTRODUCTION

Lipopolysaccharide (LPS, endotoxin ) of the Gram negative bacteria outer wall plays a central role in the pathophysiology of the sepsis syndrome[1]. It has been linked to the pathogenesis of acute pancreatitis[2]. The presence of endotoxin in blood and peritoneal fluid correlates with the severity, systemic complications, and mortality rates of acute pancreatitis[3]. Interestingly, LPS has been found in the plasma of patients suffering from severe pancreatitis at an early stage of the disease[3]. Changes resembling acute pancreatitis were also described after administration of LPS to several animal species[4]. However, little is known about the role of LPS in the induction of the acute pancreatitis.

Intracellular $Ca^{2+}$ plays a fundamental role in regu-

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lating numerous enzyme activities and mediates effects of hormones and growth factors that control a variety of cellular processes, such as muscle contraction, metabolism, cell differentiation and secretion. An increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), is an important triggering mechanism for the activation of a variety of cellular process that can alter cellular function and even lead to cell injury and death[5]. Alteration of intracellular $Ca^{2+}$ homeostasis is an early event in the development of irreversible cell injury[6]. A sustained increase in $[Ca^{2+}]_i$ has been observed after acute pancreatitis and was suggested to play a key role in the pathogenesis of acute pancreatitis[7].

Nevertheless, it is not certain whether LPS damages directly the freshly isolated pancreatic acinar cell and its cellular mechanism in cell damage. To examine the potential for LPS to exert direct effects on the function of pancreatic acinar cell, we studied the effects of LPS on calcium homeostasis of pancreatic acinar cells. Recently, microscopic measurement techniques for recording changes in $[Ca^{2+}]_i$ in single cells have become available. Change in intracellular $Ca^{2+}$ concentration can be observed by scanning confocal microscopy. Using this approach, we investigated the changes in intracellular $[Ca^{2+}]_i$ in the absence and presence of $Ca^{2+}$ in extracellular fluid in single pancreatic acinar cell exposed to LPS, so as to examine whether LPS exerted a direct damage on pancreatic acinar cells and the relationship with the disorder of calcium homeostasis of pancreatic acinar cells.

## MATERIALS AND METHODS

**Animals and materials** Male Sprague-Dawley rats (200-250 g) obtained from Experimental Animal Center of Chinese Academy of Sciences (Grade SPF II Certificate No SYXK 2002-0023) were fasted for 12 h. *Escherichia coli* LPS (WE coli 055:B5) and MTT were purchased from Sigma Co, USA. Fluo-3/AM is a product of Molecular Probes Co, USA. All other chemicals were purchased from local source at the highest purity available. HEPES buffer salt solution (HBSS) (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.13, $NaH_2PO_4 \cdot 2H_2O$ 1.0, *D*-glucose 5.5, HEPES 10, bovine serum albumin 2 g/L, minimum essence medium 2 %, *L*-glutamine 2.0, soybean trypsin inhibitor 0.1 g/L, pH adjusted to 7.4 with NaOH 4 mmol/L. Calcium–free HEPES Buffer Salt Solution, except without calcium, other ingredients were the same to those in HBSS.

**Preparation of isolated pancreatic acinar cells** Pancreatic acinar cells were isolated from male SD rats by collagenase digestion[8]. In brief, after the rat was anesthetized, pancreas was quickly removed and parenchyma was minced into small fragments and incubated in 10-mL standard buffer containing collagenase V (90 kU/L) at 37 °C, and the pancreatic fragments were digested again by collagenase under a shaking condition for 20 min in an incubator. After collagenase digestion, tissue was gently pipetted. Dispersed acini were filtered through a 150-μm nylon mesh, centrifuged 3 times each for 3 min at 10×*g* , resuspended with culture media (in HBSS, replacing bovine serum albumin 2 g/L with 10 % heated-inactivated bovine serum) and incubated with 95 % $O_2$ and 5 % $CO_2$.

**Cell culture and treatment** Pancreatic acinar cells were cultured at 37 °C in a $CO_2$ (5 %) incubator. Some cells were planted on a poly-lysine coated coverslip that was preplaced in 3.5-cm Petri dish. The coverslips were taken out from the dish after cell attachment (about 4 h) and $[Ca^{2+}]_i$ in single cell was measured. Another cells were planted in 96-well plates and cultured for 4 h, then exposed to different doses of LPS (1, 10, and 20 mg/L), and 0.1 % $Me_2SO$ (as a dissolvent of LPS and Fluo-3/AM) or cultural media as control during indicated period respectively.

**MTT assay** MTT assay was employed to assess the viable cell number quantitatively. Briefly, 100 μL of cell suspension ($1 \times 10^4$ cells) was seeded into 96-well tissue-culture plates containing LPS (1, 10, and 20 mg/L). Control cells were not treated with LPS. After treatment with these agents for indicated peroid, 10 μL MTT (terminal concentration 0.5 g/L) was added into each well, and incubated for 4 h. The formazan crystals were produced by viable cells and dissolved by $Me_2SO$, and the optical desenity (*OD*) of the solution was measured at 490 nm of wavelength. Cell viability is directly proportional to *OD* value. The viable cell number was expressed as a percentage of control cells, measured as $100\ \% \times OD_{490,\text{LPS-treated}}/OD_{490,\text{control (at 0 h timepoint)}}$.

**Measurement of $[Ca^{2+}]_i$ with Fluo-3/AM in single cell** For single-cell $[Ca^{2+}]_i$ measurement, pancreatic acinar cells were planted on poly-lysine-coated coverslips for attachment for 4 h, and then incubated with Fluo-3/AM 10 μmol/L in darkness for 30-50 min at 37 °C prior to the $[Ca^{2+}]_i$ measurement. After being rinsed twice with HBSS, the coverslips were mounted in a perfusion chamber and continuously perfused with HBSS culture medium at a rate of 1 mL/min. The vol-

ume of the chamber was about 150 μL, and the tested agents were applied by perfusion. Temperature was kept at 37 °C during the measurement. $[Ca^{2+}]_i$ measurement was performed on an Ultima™ Adherent Cell Analysis and Sorting Laser Cytometer equipped with an Olympus IMT-α inverted microscope. The cells loaded with Fluo-3/AM were excited at a wavelength of 488 nm, and the emitted fluorescence was detected at 530 nm. The data of relative fluorescence were processed by Microsoft Excel (version 2000). The fluorescence signals were not calibrated to absolute values of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was assayed by the amended method of Wu *et al*[9]. The peak of fluorescence signals were represented as $\Delta F/F_0 \times 100$ %, where $F_0$ was the resting fluorescence during the process of measuring and $\Delta F$ was the fluorescence at each time-point of scan.

**Statistical analysis** Each *n* refers to the number of separated experiments. Data were expressed as mean±SD, *t*-test was used to determine statistical significance. $P<0.05$ was considered significant.

## RESULTS

**Effect of LPS on pancreatic acinar cells** In culture media containing physiological concentration of calcium, cell viability was reduced by LPS 1-20 mg/L in a time- and dose-dependent manner, and compared with control at the same time point, the difference was significant ($P<0.05$, Fig 1).

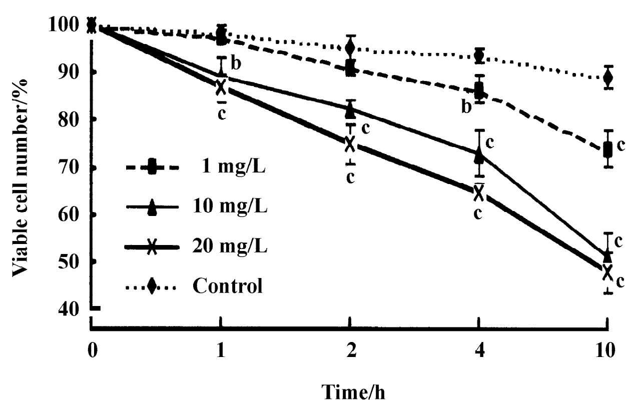


**Fig 1. Cytotoxic effect of lipopolysaccharide on pancreatic acinar cells. Results are expressed as percentage of control values (without LPS treatment) at 0 h. *n*=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group at the same time-point.**

**LPS-induced cell damage is dependent on $Ca^{2+}$** Cell mortality obviously increased after treatment with LPS 10 mg/L under physiological $[Ca^{2+}]_i$ in extracellular fluid (compared with control group, at 1 h, $P<0.05$; or at 4 h and 10 h, $P<0.01$). Removal of extracellular $Ca^{2+}$ with egtazic acid 1 mmol/L attenuated LPS-induced cell damage at 4 h ($P<0.05$) and 10 h ($P<0.01$) (compared with LPS group at the same time-point, Fig 2). The result indicated that LPS-induced pancreatic acinar cell damage was dependent on $Ca^{2+}$.

**Effect of LPS on calcium homeostasis of rat pancreatic acinar cells** $[Ca^{2+}]_i$ in pancreatic acinar cells was monitored at 10-s intervals in normal medium (during the early stage of 100 s) and then in medium containing LPS in different concentrations (5 mg/L, *n*=4; 10 mg/L, *n*=6; 20 mg/L, *n*=6). At about 100 s, LPS was added (as shown by arrow). Basal (unstimulated ) $[Ca^{2+}]_i$ in rat pancreatic acinar cells had a mild fluctuation and the average fluorescence alteration value in the basal state was 69.9 %±21.5 %. The addition of LPS 10 and 20 mg/L resulted in a significant rise in $[Ca^{2+}]_i$ (Fig 3). The rise in $[Ca^{2+}]_i$ typically peaked within

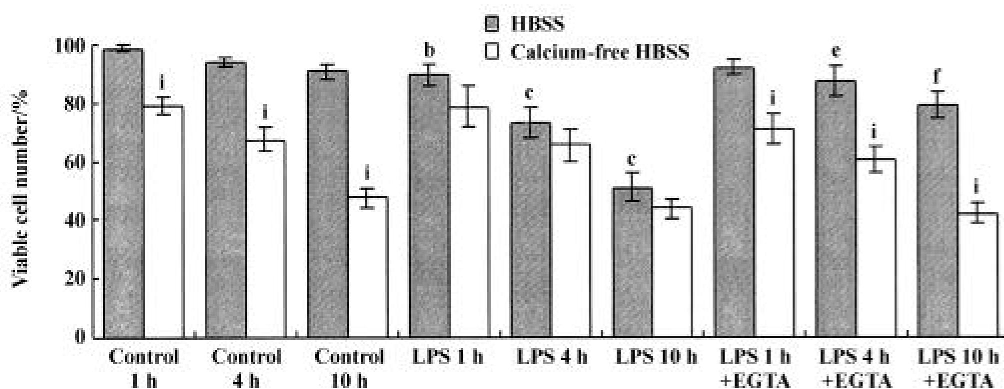


**Fig 2. Effect of LPS on pancreatic acinar cell damage in the presence and absence of calcium. *n*=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group. $^eP<0.05$, $^fP<0.01$ *vs* LPS group. $^iP<0.01$ *vs* HBSS containing calcium group.**

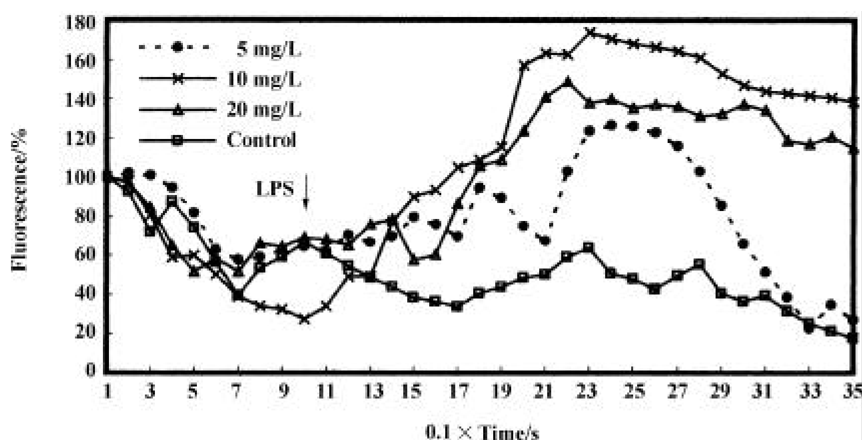


**Fig 3. Tracing of the rise in $[Ca^{2+}]_i$ elicited by LPS in rat pancreatic acinar cells. Fluorescence was shown as Δ $F/F_0$´100 %.**

150 s after stimulation with LPS 10 mg/L (the average fluorescence alteration value was 173.6 %±20.8 %), and then $[Ca^{2+}]_i$ returned to basal or lower level within 400 s (data not shown). Each final solution of LPS contained 0.1 % $Me_2SO$ vehicle. $Me_2SO$ vehicle (up to 1 %) in HBSS as control had no effect on $[Ca^{2+}]_i$.

**Origin of $Ca^{2+}$ increase in cytoplasma of pancreatic acinar cells** To examine the nature of the rise in $[Ca^{2+}]_i$ elicited by LPS, we compared responses in the absence and presence of egtazic acid. Egtazic acid 1 mmol/L was added 10 s before the addition of LPS 10 mg/L. Under these conditions, any rise in $[Ca^{2+}]_i$ should be due to release of intracellular $Ca^{2+}$. Under the physiological concentration of $Ca^{2+}$ and without egtazic acid in extracellular fluid, LPS induced a rapid, obvious rise in $[Ca^{2+}]_i$ (Fig 4A). In the absence of calcium in the bathing medium, LPS caused a mild sustained rise in intracellular calcium levels (Fig 4B). The re-addition of extracellular calcium in the medium resulted in a more immediate and remarkable rise in $[Ca^{2+}]_i$, which reached the peak value (near-dye saturation) within 100 s and maintained the value sustainedly for about 100 s, and then decreased to basal or lower level within 400 s.

## DISCUSSION

Despite considerable progress in understanding pathophysiology of pancreatitis, the mechanisms of the development of this disease remain obscure. A number of animal models of experimental acute pancreatitis have been developed and shown biochemical, morphological, and physiopathologic similarities to various aspects of human acute pancreatitis[10]. At present, rat has been chosen as the most important animal in the studies about pancreatitis because of the following traits. The conduct formed by biliary duct and pancreatic duct makes it easy to be intubated or injected to make a successful model; furthermore, there are many bioactive substances which possess extensive homology as those in human body and are easy to be contrasted with human. As a result, we dissociated pancreatic acinar cells from rats to observe their responses to stimulus.

In the present study, we observed that LPS induced calcium overload in cytoplasma and damage in intact pancreatic acinar cells. We found that, in the absence of extracellular calcium, LPS only initiated a mild and sustained rise in $[Ca^{2+}]_i$, but the re-addition of calcium in the continuous presence of LPS resulted in a more rapid and significant rise in $[Ca^{2+}]_i$, again rising to near-dye saturation, indicating that a calcium permeation pathway had been activated by the LPS exposure. These results demonstrated that LPS-induced increase in $[Ca^{2+}]_i$ resulted little from the discharge of $Ca^{2+}$ in intracellular calcium store but dominantly from the influx of extracellular calcium. This result is similar to those in other experiments. Some studies[11] indicated that pancreatic acinar cell belonged to non-excitable cell. In pancreatic acinar cell, some stimulus may induce $Ca^{2+}$ influx from extracellular fluid by the way of intracellular calcium store depletion namely through opening of a kind of calcium channel called Captive $Ca^{2+}$ Channel or Store Depletion Dependent $Ca^{2+}$ Channel (SDDCC) on cell membrane which was activated by intracellular calcium store depletion. The pathogenic factors could lead to calcium release from calcium pool by damaging the integrity of biomembrane, further activate Captive $Ca^{2+}$ Channel and cause calcium influx from extracellular fluid through the opening channel.

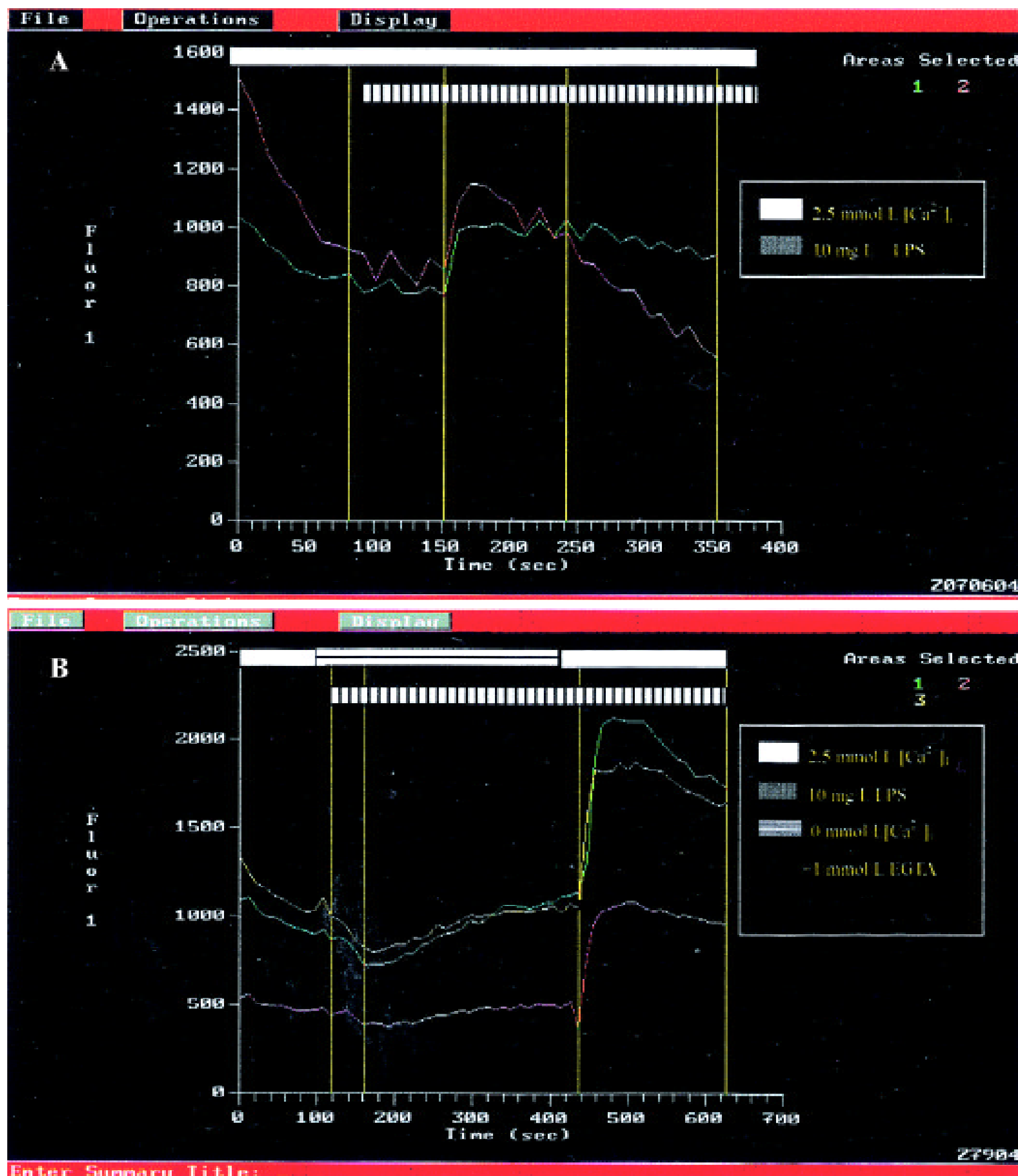


**Fig 4. Role of extracellular $Ca^{2+}$ in the response of LPS-elicited $[Ca^{2+}]_i$ rise. Fluorescence of Fluo-3/AM-loaded pancreatic acinar cells is shown. Tracings are representatives of 3 individual experiments. A) The response caused by addition of LPS (10 mg/L) to the bathing medium containing physiological concentration of extracellular calcium. B) The curves of $[Ca^{2+}]_i$ alteration from 3 different pancreatic acinar cells which were treated with LPS (10 mg/L) in the absence or presence of extracellular calcium.**

As we know, calcium serves as an important second messenger to elicit a series of signal transductions inside cells. In endothelial cells, calcium disturbance inside cells can trigger cell damage and death[12]. In our experiment, we found that LPS was able to induce the damage in intact pancreatic acinar cells directly at certain concentration and egtazic acid could attenuate the damage of pancreatic acinar cells by blocking the extracellular calcium influx. The results demonstrated that the cytotoxic effect of LPS on the isolated pancreatic acinar cells was dependent on the concentration of LPS and $Ca^{2+}$ in extracellular fluid.

The first alteration measured after exposing pancreatic acinar cells to LPS system was an increase of the $[Ca^{2+}]_i$ which appeared within several hundred of seconds. The increase of $[Ca^{2+}]_i$ preceded all the other pathological events in the progress of LPS-induced pancreatic acinar cell damage. The result suggested that the disorder of calcium homeostasis in pancreatic acinar cells was an important mediator, but not a result of

LPS-induced cell damage. Calcium overload may mediate the LPS-induced pancreatic acinar cell damage by affecting trypsinogen and phospholipase $A_2$ ($PLA_2$) activation[4], increasing the release of cytokine from activated monocytes and pancreatic acinar cells[13].

The results from our present study demonstrated that LPS could induce calcium overload in cytoplasma and its main origin was $Ca^{2+}$ influx from extracellular fluid through the opening of SDDCC probably. Intracellular calcium overload exerted an important effect on LPS-induced pancreatic acinar cell damage and egtazic acid, a $Ca^{2+}$ chelate could attenuate the damage by inhibiting $Ca^{2+}$ influx. Calcium homeostasis disorder may be one of the causes or at least an important mediator of LPS-induced pancreatic acinar cell damage. The above conclusion supports the hypothesis that calcium overload may be involved in the pathophysiology of acute pancreatitis leading to more severe pathological changes[14].

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