

# Ultrastructural aspects of acute pancreatitis induced by 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) in rats

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*Pathophysiology of acute pancreatitis (AP) has not been clearly established; nevertheless, accumulating evidence implicates highly reactive oxygen species (ROS) as important mediators of exocrine tissue damage. In this study, we used a water-soluble radical initiator, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), to investigate the consequences of oxidative stress insult to the rat pancreas. The detailed characterisation of acini ultrastructural changes in the early course (3, 6, 12, 24 h) of AAPH-induced pancreatitis (40 mg/1 kg body weight) was performed. Considerable damage to the mitochondria in acinar cells manifested by increased translucence of the matrix, partial destruction of cristae, and formation of myelin figures were noted. At the same time, focal dilation, degranulation of rough endoplasmic reticulum, and reduced number of zymogen granules was observed. The most prominent ultrastructural feature was accumulation of highly polymorphic cytoplasmic vacuoles in acinar cells. Double membrane-bound autophagosomes, different in size and shape, with sequestered organelles, autophagolysosomes, and large, empty, single-membrane-bound vacuoles were observed within the cytoplasm. The results indicate that intensive and impaired autophagy mediates pathological accumulation of vacuoles in acinar cells. The rat model of acute pancreatitis induced by AAPH is useful to investigate the early events of oxidative stress insult to the pancreas. (Folia Morphol 2012; 71, 3: 136–141)*

**Key words:** acute pancreatitis, oxidative stress, AAPH, ultrastructure, autophagy

## INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease that leads to various degrees of acinar cell damage, interstitial oedema, haemorrhage, and the recruitment of leukocytes [2, 23, 30]. Many theories about the mechanism involved in its pathogenesis have been proposed, but they are still under debate. Since the first report in the literature by Sanfey et al. in 1984 [25], many clinical and basic science studies have indi-

cated that oxidative stress and reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl free radicals are important mediators of exocrine tissue damage, which carries considerable morbidity and mortality [17, 23, 26, 27]. Accumulating evidence implicates highly reactive oxygen species as the cause of various reactions by directly attacking biomolecules (lipids and proteins) in the biologic membranes [20, 22]. Damaged acinar cells as

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well as activated neutrophils and macrophages release lysosomal enzymes, oxygen free radicals, vasoactive substances, and proinflammatory mediators. Moreover, ROS generated in the circulation might also injure the capillary endothelium, and thus accelerate the progression of pancreas dysfunction [13, 28]. In experimental models of acute pancreatitis, acinar cells have been shown to die through both necrosis and apoptosis [7]. One aspect of AP in different models, which has been of interest to some authors, is the appearance of cytoplasmic vacuoles within acinar cells [9, 29]. Vacuolisation seems to be particularly important in the context of premature trypsinogen activation by the lysosomal hydrolase cathepsin B [10, 19]. Cytoplasmic vacuoles are autophagic in origin, and this process may have important implications for the pathogenesis of pancreatitis, triggering pancreatic autodigestion [5, 18, 27]. Sequestration and degradation of a cell's own components resulting in the total destruction of acinar cells, observed in severe AP, is called type 2 programmed cell death [7]. In our experiments we performed detailed ultrastructural examination of exocrine pancreatic changes 3, 6, 12, and 24 hours after injection of AAPH. This water-soluble free radical initiator was administered to study the consequences of oxidative stress insult to the rat pancreas, with a focus on autophagy processes. We postulate that the rat model of AP induced by oxidative stress generated by AAPH is useful to investigate the early stages of AP.

## MATERIAL AND METHODS

### Experimental design

The experiments were carried out on 50 male Wistar rats, 260–380 g body weight, housed at room temperature, using a 12-h light-dark cycle. They were fed with a laboratory chow diet and fasted overnight, before the experiment, with free access to water. Care was provided in accordance with the “European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.” The procedures were performed in accordance with the institutional requirements of the Ethics Committee for Animal Care of Poland.

### Induction of acute pancreatitis

Wistar rats were divided randomly into five groups. The animals were anaesthetised with a Ketamine and Xylazine mixture, 80 mg + 10 mg/kg ip. In control group 1, physiological saline 0.5 mL was injected into the common bile pancreatic duct (CBPD). In groups 2, 3, 4, and 5, 2,2'-azobis-(2-amidinopropane) dihydro-

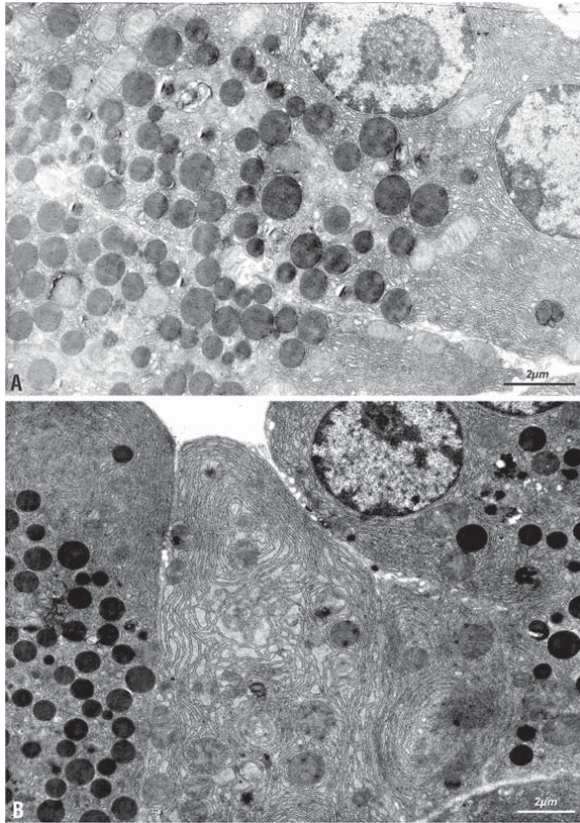
chloride (AAPH Sigma, 40 mg/1 kg body weight) was injected retrogradely into CBPD over 15 min. The proximal end of the CBPD, close to the liver, was temporarily sutured so as not to administer AAPH directly into the liver. After infusion of AAPH the catheter was removed and the duodenal loop was sutured. After observation periods of 3, 6, 12, and 24 hours, all surviving animals were sacrificed by exsanguination under anaesthesia. Each pancreas was quickly removed, blotted dry, and weighed. Small tissue probes were excised from the pancreatic head and body for light- and electron-microscopic examination.

### Ultrastructural examination

Small specimens (about 1 mm<sup>3</sup>) of pancreatic tissue (three from each animal) were immediately fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 3 hours. Next, they were postfixed in 2% osmium tetroxide for 1 hour at 4°C. The samples were dehydrated in alcohol and propylene oxide and then embedded in Epon 812. The ultrathin sections were cut from each block on a Reichert Om U3 microtome, collected on copper grids, and counterstained with lead citrate and uranyl acetate. They were then examined and photographed using a JEM 1200EX II (JEOL, Tokyo, Japan) transmission electron microscope. Fifty to sixty electron micrographs of the most characteristic changes from each group were made.

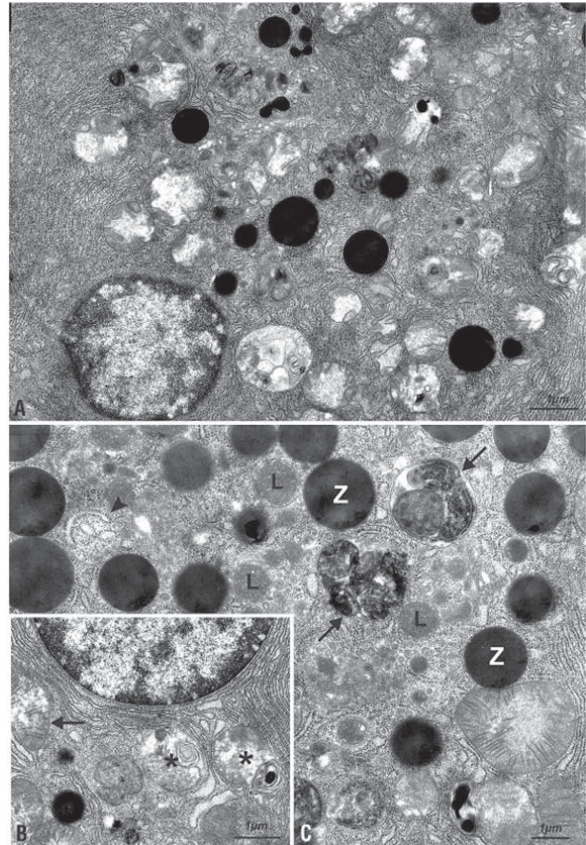
## RESULTS

The ultrastructural appearance of pancreatic acinar cells did not show abnormalities in the control group. Polarised acinar cells had abundant parallel stacks of rough endoplasmic reticulum with interspersed mitochondria in the basal cytoplasm. The supranuclear region of a pancreatic acinar cell illustrated condensing vacuoles in the Golgi region and homogeneous electron-dense zymogen granules concentrated in the apical cytoplasm. The luminal spaces were lined by short microvilli (Fig. 1A). Treatment with AAPH induced focal changes in the rat exocrine pancreas concerned with acinar cells, intercellular space, and microcirculatory vessels. Pancreata removed 3 or 6 hours after injection of AAPH showed considerable damage to the mitochondria of varied intensity. Mitochondrial swelling was a prominent finding in this group. We also noticed increased translucence of the mitochondrial matrix, partial destruction of cristae, and formation of myelin figures (Figs. 2A, B). Additionally, early ultrastructural



**Figure 1.** Normal ultrastructural appearance of the pancreas from the control group, characterised by the pyramidal acinar cells with abundant parallel stacks of rough endoplasmic reticulum, interspersed mitochondria in the spherical nuclei. Numerous well-defined mature secretory granules are seen in the intact acinar cells (A). Ultrastructural degenerative alterations of acinar cells in the middle of the figure (3 hours after AAPH induction) are illustrated. Dilatation and focal degranulation of the rough endoplasmic reticulum, mitochondrial swelling, and reduced number of zymogen granules are characteristic early features indicating cellular damage (B).

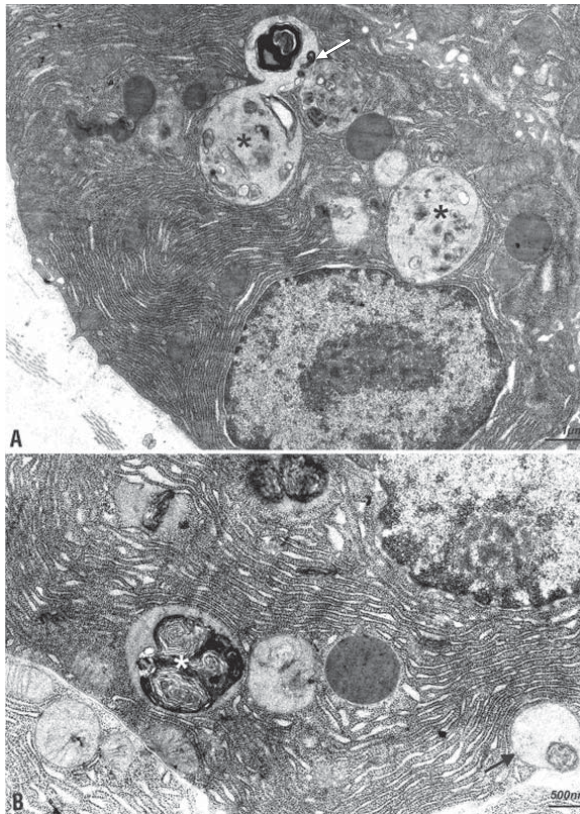
changes appreciated between 3 and 6 hours after AAPH administration revealed alteration in the secretory compartment, including mild and moderate dilatation and degranulation in the rough endoplasmic reticulum (RER), dilation of Golgi cisternae, and a reduced number of zymogen granules (Fig. 1B). However, the most distinct structural feature after AAPH treatment was cytoplasmic vacuolisation of acinar cells. Numerous autophagosomes were noted, different in size and shape, within the cytoplasm of acinar cells. Twenty-four hours after induction of pancreatitis, the autophagic process was most pronounced (Fig. 2B). Autophagosomes revealed typical characteristics with identifiable good preserved organelles, such as: RER, mitochondria, dense bodies, lysosomes, and ribosomes. Autophagolysosomes, also called



**Figure 2.** Representative electron micrographs of pancreatic tissue in AAPH-treated rats — 3 hours after injection. Changes include swollen mitochondria with increased matrix translucence and destruction of cristae. Note the undergoing formation of autophagic vacuoles containing electron dense materials, and vacuoles with concentric myelin figures. In the affected acinar cells, zymogen granules are rare, varying in size and electron density (A). Early autophagic vacuoles with recognizable mitochondria (arrow), small clumps of lamellar, membranous structures, granular deposits, and other osmiophilic amorphous patches (asterisk) are observed in acinar cells. Normal morphology of the nucleus and only slight dilation of the endoplasmic reticulum cisternae is visible (B). Autophagosomes after fusion with lysosomes form autolysosomes (arrow) in which the electron-dense sequestered material is digested (C); L — lysosome; Z — zymogen granule; phagophore — isolation membrane (arrowhead).

secondary lysosomes, not surrounded by a double membrane, in AAPH-induced pancreatitis were observed. Numerous autophagic vacuoles contained non-degraded, partially degraded, or nearly completely digested pleiomorphic material, such as amorphous masses, membranous formations, or granular elements, were visible (Figs. 3A, B). Fusion between different autophagic vacuoles was observed (Fig. 3A). Multiple deposits of cholesterol were manifested as concentric, myelinated



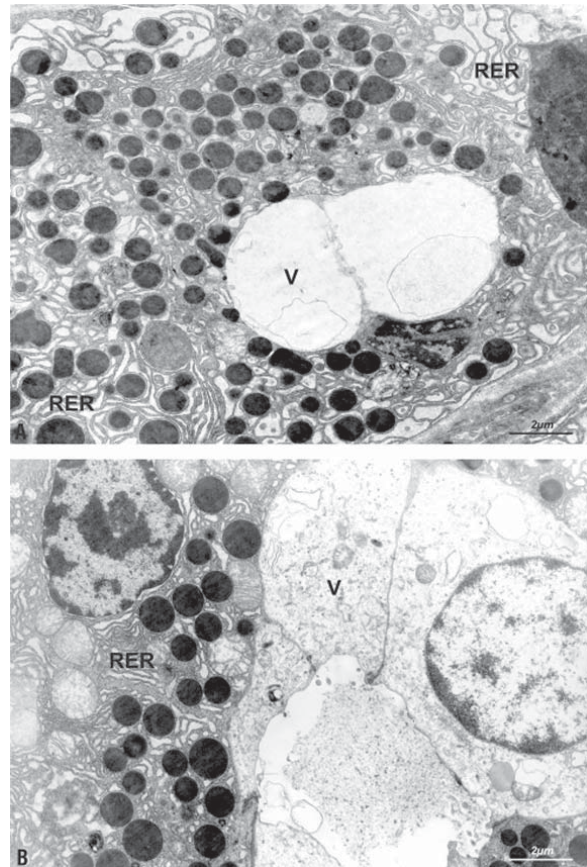


**Figure 3. A, B.** Electron micrograph demonstrating structure of autolysosomes (secondary lysosomes) in AAPH-induced pancreatitis (24 hours after induction). These organelles with nearly completely digested material, not surrounded by a double membrane, seem to be autolysosomes during advanced stage of formation (arrow). Fusion between different autophagic vacuoles (arrows) is observed (A). Cholesterol deposits visualised as multilamellar concentric, myelinated figures (asterisk). Basolateral exocytosis of autophagic vacuole (arrow) into the interstitial space (B).

membranes. At the same time, abnormally large empty vacuoles as an important cellular hallmark of early pancreatitis were present (Figs. 4A, B). Apoptotic death of acinar cells occurred only occasionally in some areas of the pancreas (Fig. 5B). It is important to underline that intercellular oedema, destruction of capillary endothelium, and inflammatory infiltration or apoptosis was only sporadically observed (Fig. 5B).

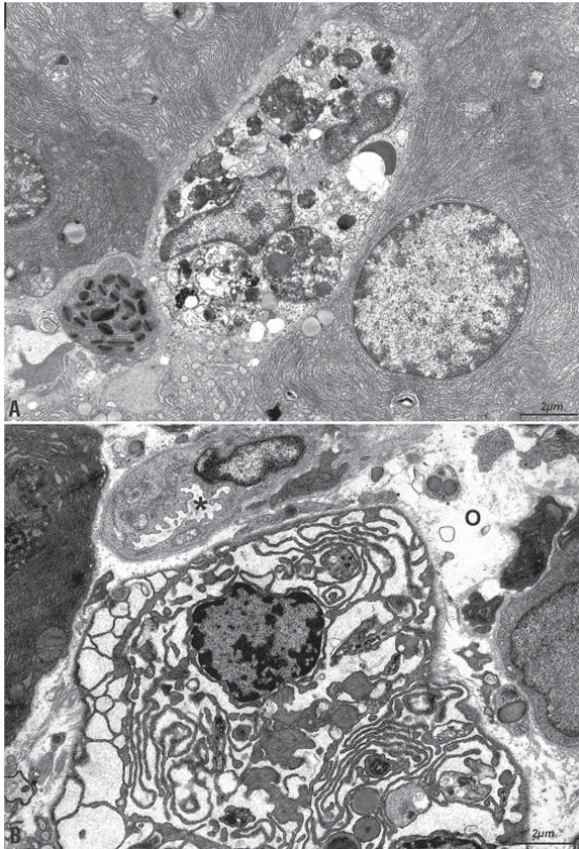
## DISCUSSION

From clinical and experimental studies, several lines of evidence suggest that highly reactive oxygen species and leading oxidative stress have been implicated in the initiation of AP [14, 22, 24, 26, 28]. The effects of various free radical scavenger treatments on AP are indirect proof for the role of ROS in the pathogenesis of this disease [16, 21]. There have been



**Figure 4. A, B.** Electron micrographs showing the intensive acinar cell vacuolisation 24 hours after AAPH induction. Abnormally large empty vacuoles as an important cellular hallmark of early pancreatitis are observed (V). Mild and moderate dilatation and degeneration in the rough endoplasmic reticulum (RER) channels is also illustrated.

numerous experimental ultrastructural studies concerning induction of AP [1, 16, 28, 29]; surprisingly, only a few studies have been performed to investigate the potential of AAPH to induce oxidative stress in vivo [11, 12]. As a consequence, the question of whether ROS act as mediator or as initiator in AP under in vivo conditions remains unanswered [24]. Acinar cell injury is regarded as the most important step in pathogenesis of AP; therefore, we applied a suitable experimental model to investigate the potential of ROS to induce morphologic alterations in vivo. AAPH, a water-soluble free radical initiator, was used for detailed ultrastructural characterisation of the early stages of AP in rats. It is important that the rate of decomposition of AAPH is determined primarily by temperature. At 37°C and pH 7 the half-life of AAPH is about 175 hours, which means that the rate of radical generation is constant for the first few hours [11, 20]. Our results demonstrate that treatment with



**Figure 5.** Granulocyte infiltration, macrophage invasion into the pancreatic parenchyma (A), and apoptotic death of acinar cells (B) only occurred sporadically in some areas of the pancreas (24 hours after AAPH induction). The interstitial tissue shows marked oedema (O), destruction of capillary endothelial cells (asterisk), and luminal narrowing (B).

AAPH induced focal changes in acinar cells, the main cell type of the exocrine pancreas. Electron microscopic investigations revealed considerable damage to the mitochondria and distinct vacuolisation of acinar cells. Increased translucence of the matrix, partial destruction of cristae, and formation of myelin figures within injured mitochondria started at the 6-hour time point after AAPH induction and were more pronounced after 24 hours. Moreover, changed mitochondria were observed inside autophagic vacuoles. Such mitochondrial dysfunction may be caused, via lipid peroxidation, by free radicals generated after AAPH application [8]. It is well known that mitochondria are highly dynamic organelles, crucial for energy production; thus changes in their morphology and alterations of functions are linked to multiple acute diseases, including AP [6, 9, 18]. The results presented here visualise a very active autophagy process manifested by the appearance of different sized and

shaped autophagic vacuoles within the pancreatic acinar cells. Nevertheless, autophagic flux was impaired in our model of experimental pancreatitis induced by AAPH. Our findings are in agreement with other observations where the potential of ROS to induce morphologic alterations of AP was investigated, but the role of autophagy and its possible defects in pancreatitis is only starting to be elucidated [12, 19, 24]. Studies provided by Gucovsky et al. [8] revealed that autophagy, the principal cellular degradative process, is impaired in pancreatitis due to inefficient lysosomal function and mediates pathological accumulation of vacuoles in acinar cells. In addition, recent studies by Kloppel et al. [15] have demonstrated intracellular vacuoles during the early course of AP in humans. Autophagy (specifically macro-autophagy) is an evolutionarily conserved catabolic process that may occur as a general phenomenon but probably plays a cardinal role in the cellular adaptation to stress [3, 4]. This process may have important implications for the pathogenesis of pancreatitis because the lysosomal enzyme cathepsin B can activate trypsinogen and may, in this way, trigger pancreatic autodigestion. Autophagy eliminates dysfunctional or damaged mitochondria, thus counteracting degeneration and inflammation and preventing cell death [6, 21]. Taken together, we agree with other authors and conclude that the combined autophagic, lysosomal, and mitochondrial dysfunctions can mediate the pathologic responses of pancreatitis. It is important to underline that oxidative stress generated by AAPH in our study changed acinar cell morphology and function without causing their death. The death of pancreatic cells that occurred only occasionally in some areas of exocrine pancreas was obviously due to apoptosis.

The obtained results indicate that:

- the rat model of AP induced by application of AAPH is useful to investigate the early events of oxidative stress insult to the pancreas;
- pathological accumulation of empty megavacuoles in acinar cells mediated by impaired autophagy is an important cellular hallmark of early pancreatitis;
- the detailed ultrastructural characterisation of the early stages of AAPH-induced AP in the present study may be useful in further investigations of disease pathogenesis.

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