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PROOXIDATIVE MECHANISM OF ACTION OF PERACETIC ACID IN CELLULAR MODEL OF ACUTE PANCREATITIS

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PhD Thesis

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2017

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This dissertation is dedicated to my Parents.

Words cannot express my gratitude and love to both of You.

1. SUMMARY

Acute pancreatitis is a common and potentially lethal disease, with no current therapies directed to the molecular mechanism of its pathogenesis. Gallstones and alcohol abuse causes about 75% of acute pancreatitis. It has been previously shown that ethyl alcohol abuse lead to Reactive Oxygen Species (ROS) formation in acinar cells of pancreas, that in turn enhance the oxidative stress of the acinar cell. Oxidative stress underlies in premature digestive enzymes activation and self-digestion of whole pancreas.

Main science resources highlight acetaldehyde and acetic acid as a final product of ethyl alcohol metabolism. In this study I would like to present the new theory, which states that acetic acid as a physiologically occurring alcohol metabolite reacts with hydrogen peroxide (normal product of respiratory chain activity) and leads to formation of peracetic acid. Such reaction is well known to simple laboratory chemistry, but was never concerned to be present in biological structures. The hypothesis of my study assumes that peracetic acid oxidized SIRT3 - mitochondial deacetylase, result in SIRT3 deactivation. That leads to hyperacetylation, and dysfunction of mitochondrial proteins for example Hsp60 chaperon preventing cell from heat shock and wrong protein folding, and MnSOD - scavenger enzyme which is protecting the cells from potential damage caused by excessive amounts of ROS (oxidative stress). This in turn lead to increasing the ROS generation, and oxidative stress that has been shown to take part in digestive enzymes premature activation.

Obtained results partially confirmed my assumptions, and shown that peracetic acid has more prooxidative effect on AR42J acinar cells, than acetic acid, and hydrogen peroxide. These results can provide a great start to the new reasoning of pathogenesis of acute pancreatitis and suggests different molecular mechanism of the process of degradation of the pancreatic tissue during inflammation, suggesting peracetic acid as a final metabolite of ethyl alcohol metabolism.

1. BACKGROUND

1.1. The role and anatomy of pancreas

Pancreas is a glandular organ located in abdominal cavity, directly behind the stomach and next to the small intestine. The pancreas has the dual function of secreting hormones into the blood stream (endocrine) and secreting digestive enzymes through pancreatic ducts (exocrine), which makes it a part of both gastrointestinal and endocrine system.

The pancreas is about 15cm long. Anatomically, it is divided into the head, neck, body, and the tail of pancreas (Fig.1). The head is surrounded by the duodenum in its concavity and it is surrounded by two blood vessels, the superior mesenteric artery and vein. The portion of the organ that lies anterior to the aorta is thinner than the adjacent portions of the head and body of the pancreas. This region is sometimes designated as the neck of the pancreas and marks the junction of the head and body. The neck is about 2,5 cm long and lies between the head and the body, and in front of the superior mesenteric artery and vein. On the right it is grooved by the gastroduodenal artery. The body is the largest part of the pancreas and lies behind the pylorus, at the same level as the trans-pyloric plane. The common bile duct passes through the head of the pancreas to join the main duct of the pancreas near the duodenum. The close proximity of the neck of the pancreas to major blood vessels posteriorly (the superior mesenteric artery, superior mesenteric-portal vein, inferior vena cava, and aorta) limits the option for a wide surgical margin [1].

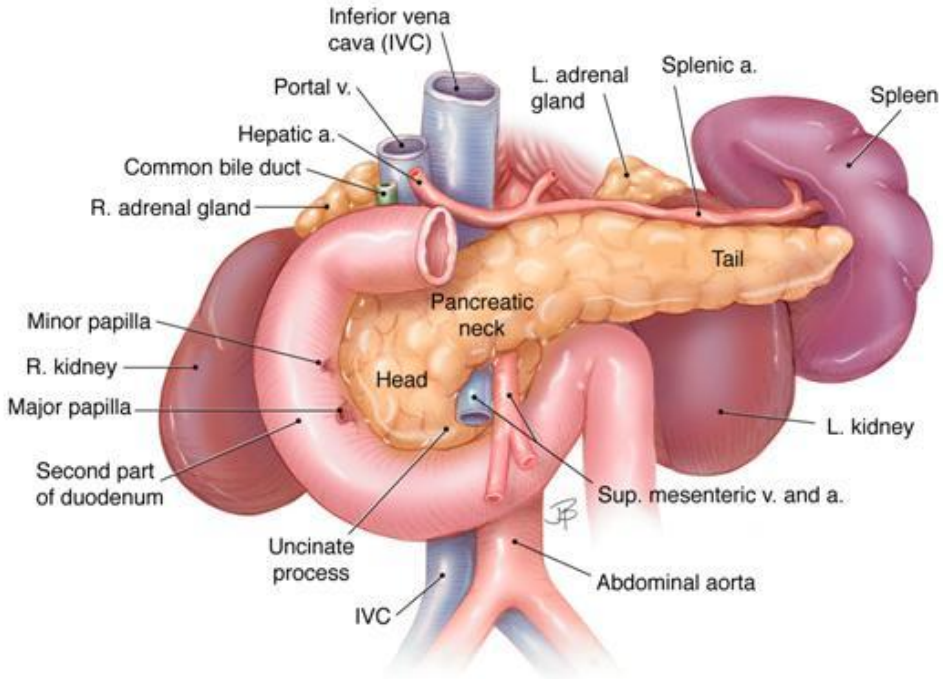

Fig.1 | Anatomic relationships of the pancreas with surrounding organs and structures [65].

1.2. Endocrine and exocrine pancreas

The adult pancreas is composed of exocrine (acinar cells and ducts) and endocrine compartments (α-, β-, δ-, ε-, and PP-cells). Each of the five endocrine cell types synthesizes and secretes one hormone: glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells), ghrelin (ε-cells), and pancreatic polypeptide (PP-cells) [4-5]. Endocrine part of pancreas synthetize and secrete insulin, glucagon, somatostatin and pancreatic polypeptide into the blood to control energy metabolism and storage throughout the body [2]. Using a microscope the

endocrine part of pancreatic tissue can be seen under staining as lightly-stained clusters of cells, called pancreatic islets. Only about 5% of the pancreas is comprised of endocrine cells. These cells are clustered in groups within the pancreas and look like little islands of cells under a microscope. These groups of pancreatic endocrine cells are known as pancreatic islets or more specifically, islets of Langerhans and are responsible for hormone secreting directly to the blood stream [6].

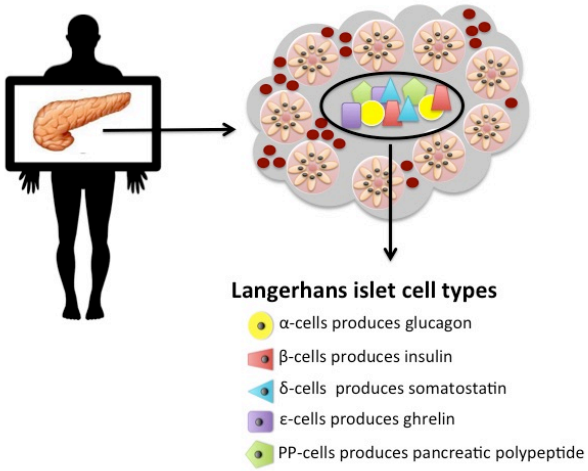

Fig.2 | Islet of Langerhans

Islets of Langerhans constitute the endocrine part of the pancreas, situated in pancreatic tissue. They consist of many cell types responsible for different hormones secretion. Hormones are released directly to the blood stream.

The other one, exocrine part, is responsible for formation and secretion of digestive enzymes (proteases, lipases and amylases) into the duodenum to catalyze the hydrolysis of food constituents [3]. The exocrine pancreas consists

of 3 distinguishable epithelial cell types: acinar (constituting 85% of the whole organ mass) centro-acinar (CAC) and ductal cells. The acinar cells are responsible for synthesizing, storing, and secreting digestive enzymes. These exocrine cells release their enzymes into progressively larger tubes (called ducts) that form together the main pancreatic duct. The main pancreatic duct runs the length of the pancreas and drains the fluid containing newly synthesizes enzymes into the duodenum where they assist in the digestion of food constituents [7].

It is important to point out that there are important interrelationships between the endocrine (islets of Langerhans) and exocrine pancreas, and that the dysfunction of one of them causes the whole organ damage. Anatomic studies demonstrate that the blood flow from the endocrine pancreas enters the capillaries of the exocrine tissue surrounding each of the islets before entering the general circulation [8]. The hormones from the islets of Langerhans include insulin, amylin, glucagon, somatostatin and pancreatic polypeptide are delivered in very high concentrations to the exocrine cells. The influence of high hormones concentrations in general blood circulation of pancreas remains unclear, but it has been shown that the acinar cells of the pancreas have insulin receptors that are involved in regulation of digestive enzyme synthesis of the exocrine pancreas [9,10].

1.3. Pancreatic acinar cells

The functional unit of the exocrine pancreas is composed of an acinus and its draining ductile. Pancreatic acinar cells produces and secretes most of the digestive enzymes that are involved in food digestion in the small intestine. They also have the greatest rate of protein synthesis of any mammalian organ, because of its highly developed endoplasmic reticulum (ER) system combined with mechanisms to modify and transport newly synthesized proteins through the secretory pathway [11,12]. In addition to its main role, the ER of acinar pancreatic cells is a major storage site of intracellular calcium, which is a regulator of stored digestive enzymes secretion into the pancreatic ductal system. When the calcium is released into the cytoplasm it mediates the stored digestive enzymes and released them [13].

The acinar cells can have different shapes: tubular, spherical, or some other, irregular form. Basolateral membrane is rich in hormones and neurotransmitters receptors that regulate the digestive enzymes secretion. The basal part of acinar cell contains nucleus as well as abundant rough endoplasmic reticulum (RER) for protein synthesis. The apical region is rich in zymogen granules that are storing the inactive forms of digestive enzymes. The surface of apical part of cell possesses also microvilli, that form together with cytoplasm and filamentous actin meshwork that is involved in exocytosis of the contents of the zymogen granules [14,15].

Tight junctions between acinar cells result in selective passage of large molecules, such as digestive enzymes and provide paracellular transport of ions and water [16]. There are also specialized areas of the plasma membrane between acinar cells called gap junction, which are acting as a pore selectively allowing small molecules (about 500-100 Da) to pass between cells. This gap junction makes the electrical and chemical communication possible [17]. For example calcium signaling, that is the main pathway in digestive enzymes secretion of acinar cells is controlled by gap junctions [18,19].

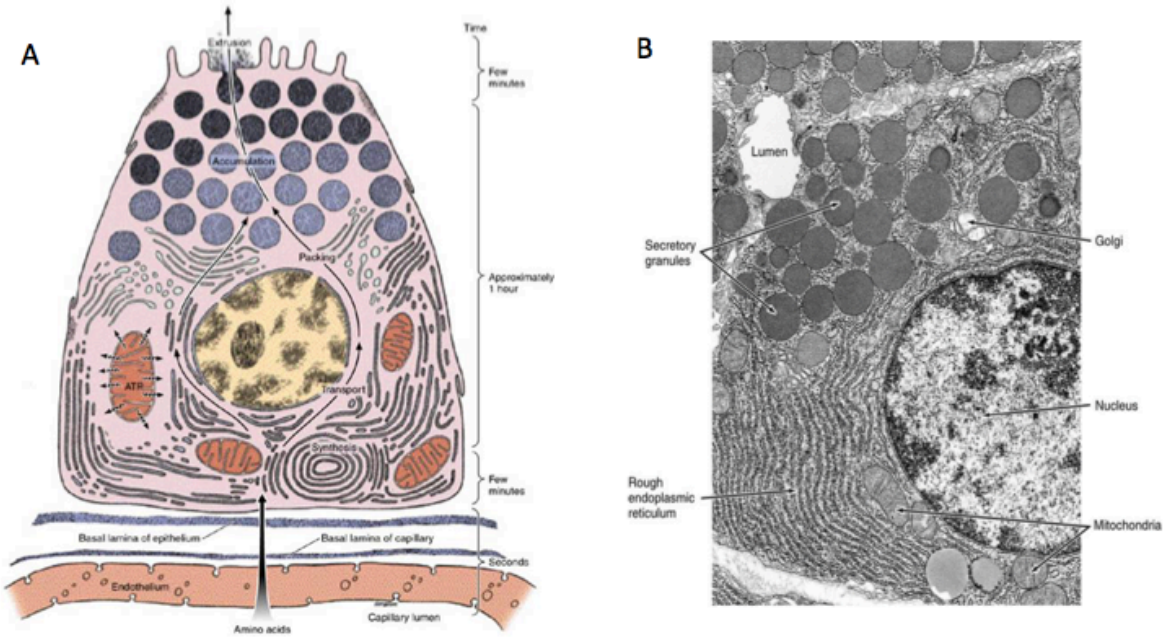

Fig.3 | Diagram of a pancreatic acinar cell (A) and ultrastructure of acinar and duct cells of the exocrine pancreas (B) [20].

The first diagram (A) shows its evident polarity, with abundant basal rough endoplasmic reticulum. The Golgi complex and zymogen granules are in the apical region. To the right is a scale indicating the approximate time necessary for each step.

The pancreatic acinar cell (B) has prominent basally located rough endoplasmic reticulum for synthesis of digestive enzymes (and other proteins) and apically located zymogen granules for storage and secretion of digestive enzymes. The zymogen granules undergo exocytosis with stimulation of secretion. The secretion is into the lumen of the acinar formed by the apical surfaces of the acinar cells with their projecting microvilli. Pancreatic duct cells contain abundant mitochondria for energy generation needed for its ion transport functions.

Digestive enzyme transcripts constitute around 80% of all acinar cell mRNAs [21]. As shown in Figure 3A, due to hydrophobic signal sequences on N-terminus the enzymes, when translated, are transported into the endoplasmic reticulum where they are post-translationaly modified (including disulfide bridge formation, phosphorylation, sulfation and glycosylation). Than they undergo

further modifications in the Golgi complex- enzymes undergo concentration and packaging into highly specialized storage structures called zymogen granules [22].

1.4. Activation of zymogen granules

Proteolytic enzymes are synthesized as inactive precursors called zymogens to prevent unwanted protein degradation. Due to neurohumoral stimulation, the receptor situated on the surface of acinar cells recognize the rising level of cytosolic calcium and leads to secretion of proenzymes situated in zymogen granules into the lumen and pancreatic duct. This phenomenon is possible by exocytosis occurs by the actin-myosin moves system, that transport granules to apical region of acinar cell where they fuse with plasma membrane [23]. Then they get activated in duodenum through a cascade of enzymatic reactions (Fig.4).

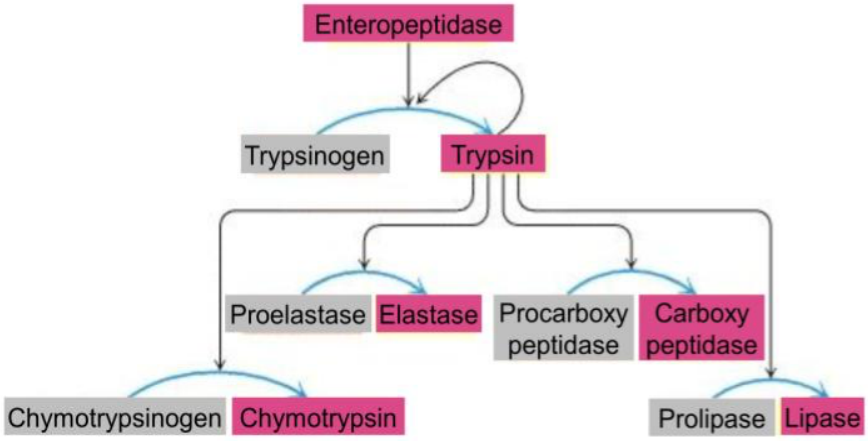

Fig.4 | Activation cascade of enzymes in duodenum [24].

Enteropeptidase activates trypsinogen by hydrolysis yielding trypsin that catalyses the activation of other proenzymes such as procarboxypeptidase (to carboxypeptidase), chymotrypsynogen (to trypsinogen), proelastase (to elastase) [25].

Proteins secreted at the apical membrane enter the pancreatic duct system and are conveyed to the intestinal lumen. Solubilized in an aqueous, bicarbonate solution secreted by pancreatic duct cells, the enzymes reach the duodenum, where the gastric acid is neutralized by the alkaline secret thus creating an environment allowing effective enzyme activation. First, enterokinase cleaves trypsinogen into active trypsin, which again converts all remaining enzyme precursors into their active forms thereby priming the breakdown of carbohydrates, proteins and lipids in the chyme to ensure nutrient absorption by the enterocytes of the small intestine.

1.5. Acute pancreatitis

Acute pancreatitis (AP) is a common and potentially lethal disease, with no current therapies directed to the molecular mechanism of its pathogenesis. AP is an acute inflammatory process of the pancreas, characterized by steady, acute abdominal pain of varying severity, often radiating from the epigastrium to the back that can range from mild interstitial pancreatitis to severe pancreatitis with pancreatic necrosis and concomitant multiorgan failure. Most cases of pancreatitis are identified by a careful history and physical examination. Despite advanced evaluation, the cause is not apparent in about 10% of cases. The etiology of recurrent acute pancreatitis appears to be multifactorial, with genetic and environmental influences playing a significant role [26].

The etiology of the pancreatitis presents as major cause of this disease, the alcoholism and the gallstone but the risk factor can be also trauma, drugs infections and hereditary factors. Gallstones or alcohol abuse causes about 75%

of pancreatitis. These risk factors are common in the actual society and are determining to the increase pancreatitis incidence, and because of this, novel molecular targeted therapies are urgently needed [27,28].

The pathogenesis of acute pancreatitis is still not well elucidated, but most hypotheses are based on the concept of a premature activation of digestive enzymes situated in zymogen granules of acinar cell in pancreas, leading to autodigestion of whole organ by tissue necrosis [29]. There are some events identified by previous studies in experimental disease models or with isolated cells. After alteration of physiological properties of acinar cells, change their functions and lead to cell injury. A number of studies suggest that inflammation, parenchymal acinar cell death by necrosis, and inappropriate intracellular activation of digestive enzymes is crucial in pathophysiologic process of AP. However, the molecular mechanism and key participants that mediate these processes remain unknown.

Acute pancreatitis begins in pancreatic acinar cells after a primary injury promotes pancreatic enzyme activation - primarily trypsin. Subsequently, the trypsin activates other enzymes those turn begin to digest the pancreatic tissue (as shown in Figure 4), whose content leaks into the abdominal cavity, causing cytokine release, arachidonic acid metabolite-secreting leukocytes, activation of the immune system, coagulation and fibrinolysis. The cascade of digestive enzymes activation leads to self-digestion of the gland. The cytokines activates the inflammatory pathway, resulting in the increase of adhesion molecules, neutrophils infiltrate, production of Reactive Oxygen Species (ROS) and several inflammatory molecules such as Prostaglandin E2 (PGE2), Tromboxan A2 (TXA2), Platelet Activating Factor (PAF). On the other hand, the release of these mediators leads to Systemic Inflammation Response Syndrome (SIRS). The increase in vascular permeability results in thrombosis and hemorrhage and can lead to pancreatic ischemia and necrosis. Increased vascular permeability can lead to bacterial translocation into the pancreatic bed and result in infected pancreatic necrosis, a life-threatening complication of AP. In severe cases,

systemic inflammatory response syndrome, renal failure, shock, myocardial stress, fever, or acute respiratory distress syndrome may develop [30,31].

1.6. Alcohol induced acute pancreatitis

The alcohol pancreatitis can have a slight different molecular background than described above. Ethyl alcohol stimulates the release of secretin and cholecystokinin, which are the major catalyst to pancreatic secretion. There are many research defined a number of alcohol-dependent biochemical changes in acinar cell of pancreas, for example sustained level of intracellular calcium, activation of the mitochondrial permeability transition pore, colocalisation of lysosomal and pancreatic digestive enzymes, reticulum stress, impairment in autophagy [32]. These changes encouraged to greater understanding of the mechanism by which ethanol predisposes acinar cell to damage and injury.

Ethanol has been shown to affect many pathways and functions of acinar cells, and the toxic effect of ethyl alcohol and its metabolism by-products sensitize the pancreas. Despite the long-standing recognition of ethanol-induced pancreatitis, the molecular mechanism for its initiation and progression is still not elucidated. There are some pathways, which were pointed to be crucial in pathogenesis of AP (Fig.5).

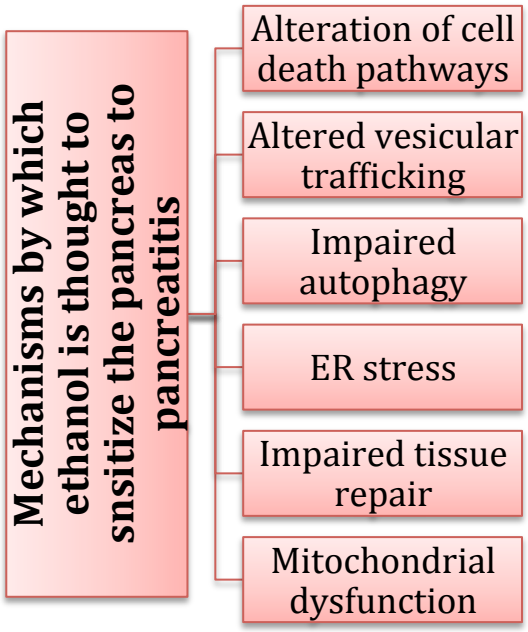

Fig.5 | Ethanol has been shown to affect a number of pathways and functions important in acinar cells

Alteration of these pathways may individually or cumulatively sensitize the pancreas, and lower the threshold of the pancreas to the development of overt pancreatitis

Many of the deleterious effects of ethanol are attributed to the by-products produced during its metabolism. The pancreatic acinar cells have the ability to metabolize ethanol by both oxidative and non-oxidative pathways. Ethanol's toxicity is mediated through the action of ethanol itself or through oxidative and non-oxidative ethanol metabolism [33].

Non-oxidative metabolism is accomplished by number of enzymes, the most important being the fatty acid ethyl ester (FAEE) synthases [34]. This type of metabolism lead to combine ethyl alcohol with fatty acids (FA) to create FAEEs, which reactivity in acinar cell is relatively high. Thus, because the oxidative metabolism of ethanol in the pancreas is relatively low, the non-oxidative metabolism of ethanol may be important and the production of FAEEs,

and their toxic effects, may be emphasized. In fact, a study of individuals who were intoxicated at the time of death revealed that the concentration of FAEEs in the pancreas was higher than in any other organ [35].

Two enzymes catalyze oxidative metabolism of ethyl alcohol: alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP 2E1). In oxidative metabolism alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde followed by chemical transformation of acetaldehyde to acetate by isoform 2 of aldehyde dehydrogenase (ALDH2) located specifically to mitochondria. This metabolism also depletes oxidized nicotinamide adenine dinucleotide (NAD^+) level while increasing level of reduced nicotinamide adenine (NADH) because ADH and ALDH are both NAD^+-consuming enzymes producing NADH [36]. Both enzymatic reaction of oxidative metabolism of ethyl alcohol by ADH or CYP 2E1 produces reactive oxygen species (ROS) and acetaldehyde. Recent findings have suggested that ethanol induces mitochondrial dysfunction via a reduction of the ratio of oxidized to reduced nicotinamide adenine dinucleotide, a mechanism distinct from the effects of cholecystokinin hyperstimulation which are mediated by increasing cytosolic calcium ($[Ca^{2+}]_c$) [37, 38].

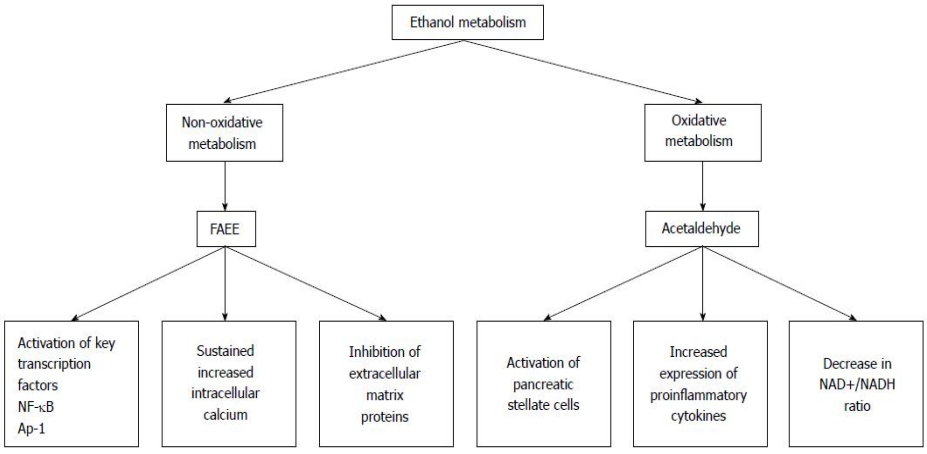

Fig.6 | The consequences of oxidative and non-oxidative metabolism of ethyl alcohol in acinar cells of pancreas [39].

Metabolism of ethanol by both of these pathways has been shown to cause a number of changes that can predispose the pancreas to acute pancreatitis.

Because the by-products of ethanol metabolism have been demonstrated to cause toxicity in other organs, a great deal of work has been performed investigating the actions of the various ethanol metabolites on the pancreas.

Oxidative metabolism of ethanol has also been shown to have deleterious effects on mitochondria. Those organelles are responsible for ATP production, needed to perform all cellular functions. Their damage can lead to cell dysfunction or even death by either apoptosis or necrosis. Mitochondrial membrane permabilisation is a trigger that initiates both necrosis and apoptosis [40]. It leads to loss of mitochondrial membrane potential (Δψm) by opening the mitochondrial permeability transition pore (MPTP) that allows the substances up to 15 000 Da to enter the mitochondrial matrix and disrupt ATP production. Using isolated mouse acinar cells, as well as, in vivo and ex vivo models of pancreatitis it has been shown that ethanol treatment reduces the Δψm and converts the normal transient decrease in Δψm caused by treatment with physiologic concentrations of cholecystokinin (CCK) to a sustained decrease in Δψm. The sustained decrease in Δψm results in reduced cellular ATP concentrations and necrosis [41] that leads to adenosine triphosphate (ATP) depletion, inability to maintain ionic gradients across the plasma membrane, and ultimately necrosis. Mitochondrial permeabilization also triggers the apoptotic pathway through release of the mitochondria resident protein cytochrome C - once in the cytosol, cytochrome c interacts with and activates caspases, leading to the downstream apoptotic events. Further studies revealed that the ethanol-induced effects on Δψm were dependent on the decreased NAD^+/NADH ratio associated with the oxidative metabolism of ethanol and its by-product acetaldehyde, and not dependent on calcium [42,43].

Metabolism of acetaldehyde is first carried out by aldehyde dehydrogenase-2 (ALDH2) a NAD^+-requiring enzyme residing on the inner mitochondrial membrane. Thus, metabolism of acetaldehyde by ALDH2 depletes NAD^+ and increases the concentration of NADH. There are some speculations

that the decreased mitochondrial NAD^+/NADH ratio and reduced Δψm is a result of the metabolism of acetaldehyde to acetate.

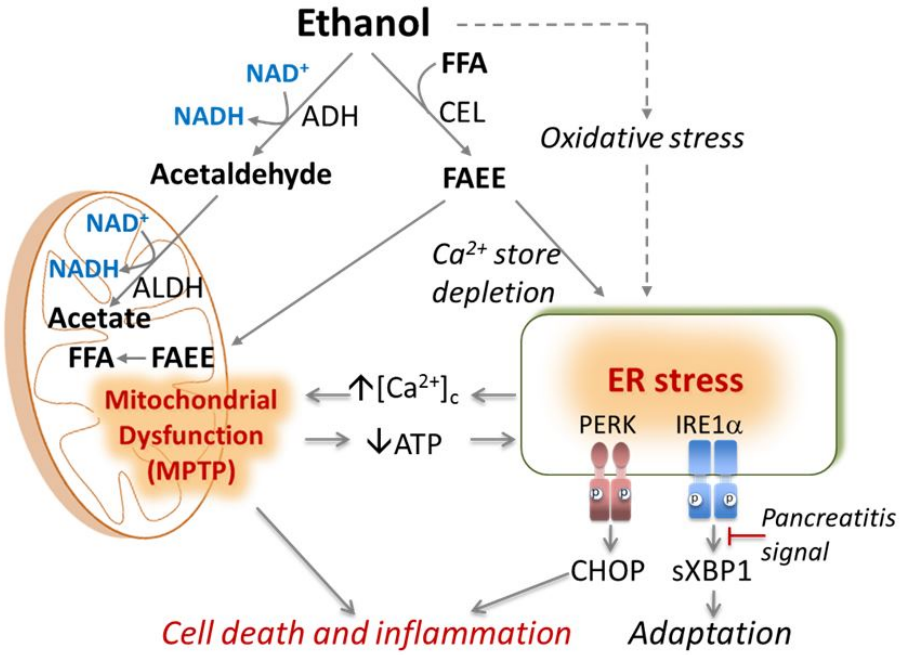

Fig.7 | Schematic diagram displaying proposed mechanisms of ethanol-mediated AP [44].

In acinar cells of pancreas, ethanol can disrupt mitochondrial function via two pathways (Fig.7). Oxidative metabolism of ethanol to acetaldehyde, via alcohol dehydrogenase (ADH), and to acetate, via aldehyde dehydrogenase (ALDH) in the mitochondria, decreases cellular NAD^+/NADH balance. Fatty Acid Ethyl Esters (FAEEs) are esterification products of fatty acids and ethanol via FAEE synthases including carboxylester lipase (CEL). Accumulation of FAEEs elicits Ca^{2+} depletion from the endoplasmic reticulum (ER) and other cellular

stores leading to sustained elevations of $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} overload. Furthermore, accumulation of FAEEs in mitochondria leads to release of fatty acids. Both altered NAD^+/NADH ratios and $[Ca^{2+}]_c$ overload can lead to opening of the mitochondrial permeability transition pore (MPTP), what in turn affect mitochondrial depolarization, ATP depletion and cellular necrosis. Besides ethanol effects on mitochondria, ethanol-induced oxidative stress alters ER redox status and elicits chronic ER stress and compromised ATP production [44].

1.7. Oxidative stress in ethanol-induced acute pancreatitis

ROS are highly reactive compounds that potentially are harmful to cell membranes, intracellular proteins, and DNA. Oxidant stress results from an balance disturbance between the production of ROS and the antioxidant mechanisms (glutathione and the enzymes glutathione peroxidase, superoxide dismutase, and catalase) within the cell. This may be the result of the production of ROS during oxidation of ethanol by CYP2E1 and acetaldehyde-induced depletion of the ROS scavenger glutathione. Oxidant stress is thought to destabilize zymogen granules and lysosomes, potentially increasing the risk of intra-acinar activation of digestive enzymes [44]. In the light of these reports, it seems to be appropriate to examine more carefully the influence of ROS in pathogenesis of acute pancreatitis.

Intracellular ROS are produced in mitochondria as a by-product of normal cellular metabolism [45] and in the cytoplasm via the xanthine oxidase and reduced nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase (NOX) pathways [46]. Under aerobic conditions, ROS are generated as by-

products of the oxygen metabolism during oxidative phosphorylation in the mitochondria. From the other hand, oxidative stress can be result from exposure to a variety of environmental agents. External sources of ROS are for example: radiation, UV light, chemical reagents, pollution, cigarette smoke, drugs and ethanol [47].

Reactive oxygen species (ROS) are physiological compound, produced as a normal metabolite and plays important roles in cells signaling and homeostasis. ROS can modulate mitochondrial functions via regulating electron transfer chain enzymes and mitochondrial membrane potential and are crucial for various cellular processes, including cell growth, apoptosis, cell adhesion and immune responses so they are essential for a proper functioning of living cells. However, in excess, can cause the oxidative damage of cellular macromolecules including proteins [48], lipids (such as the phospholipids that form cellular membranes), and DNA, thus compromising numerous intracellular pathways, cellular integrity, and causing genetic alterations [49]. This can ultimately culminate in cell death or neurodegeneration [50].

In order to prevent ROS-induced cellular damage organisms have a variety of defense mechanisms that can be referred to as the endogenous antioxidant system [51]. Endogenous antioxidants (which can either inhibit the formation of ROS or promote the removal or scavenging of free radicals and their precursors) can be further subdivided in two major groups, non-enzymatic and enzymatic. Endogenous non-enzymatic antioxidants include thiols and GSH [52].

Enzymatic antioxidants include:

- SODs, which inactivate $O_2^{-\bullet}$ and exist as copper/zinc-containing SODs in the cytosol or manganese-containing SODs in the mitochondria [53];
- catalase, an iron-containing enzyme that detoxifies hydrogen peroxide (H_2O_2) by reducing it to water [54]);
- GPx system, which encompasses the enzymes GPx and glutathione

reductase (GR) and uses GSH and NADPH as co-factors. GPx reduces hydrogen peroxide and other organic peroxides at the expense of GSH, which is in turn oxidized to form glutathione disulfide (GSSG). GSH is regenerated by GR with the consumption of NADPH [55].

Oxidative stress in acute pancreatitis resulted in excessive production of ROS and RNS leading to the impaired ability of tissue to detoxify above intermediates. ROS and RNS are accumulated in the tissue leading to its damage .The harmful effects of ROS and RNS in acute pancreatitis have been confirmed in previous studies [51]. Oxidative and nitrosative stress arises during the first phase of acute inflammation because of the generation of reactive oxygen and nitrogen species - hydroxyl radical, superoxide anion, hydrogen peroxide, peroxynitrite - that lead to glutathione depletion and oxidation, protein carbonylation, and formation of reactive carbonyl species, such as aldehydes (malondialdehyde and 4-hydroxy-2-nonenal), α , β -unsaturated ketones (cyclopentenone prostaglandins), and isoprostanes generated from lipid peroxidation and arachidonic acid metabolism, as well as nitrated and nitrosylated proteins. Glutathione (GSH) depletion in the pancreas is a hallmark of the early course of acute pancreatitis and it contributes to the severity of the disease [52].

Ethanol is metabolized in two steps. First, ethanol is converted into acetaldehyde by the enzymes alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1). Acetaldehyde is then further oxidized to acetate by the enzyme acetaldehyde dehydrogenase (ALDH). ADH-mediated ethanol metabolism results in the generation of reducing equivalents in the form of reduced nicotinamide adenine dinucleotide (NADH) and acetaldehyde, whereas ethanol oxidation by CYP2E1 leads to the production of acetaldehyde, but also to the generation of reactive oxygen species (ROS). NADH is reoxidized to NAD^+ in the mitochondria, which may further increase the formation of ROS (Fig.8).

Metabolism of alcohol leads also to H_2O_2 production, which in turn can activate tumor necrosis factor- α (TNF- α) and interleukin-1β (IL1β) - primary cytokines in acute pancreatitis because they initiate and propagate most of the consequences of the systemic inflammatory response. They initiate the inflammatory cascade by activating mitogen-activated protein kinases (MAPKs) and nuclear factor- κB (NF- κB), which induce the release of chemokines and other cytokines, and a positive feedback loop, which upregulates their own expression.

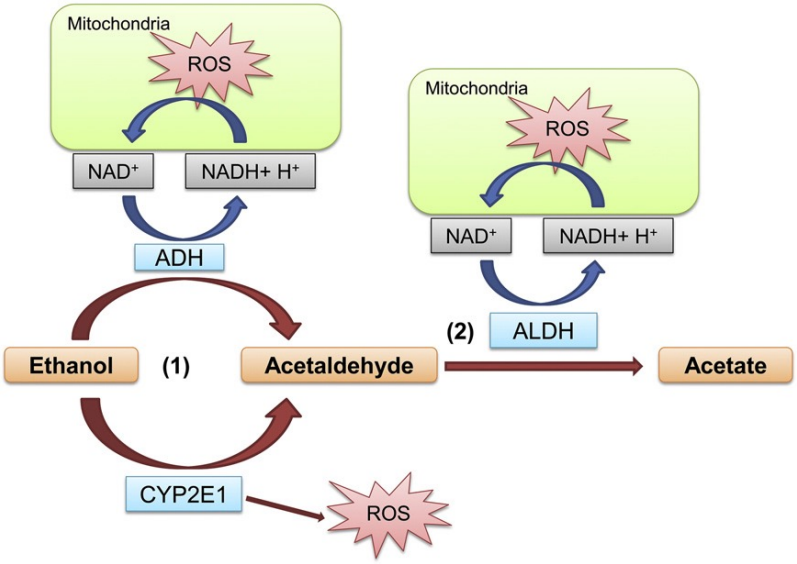

Fig. 8 | The oxidative metabolism of ethanol cause oxidative stress [56].

Ethanol and choledocholithiasis constitute the most frequently mentioned risk factors leading to acute inflammation of the pancreas (pancreatitis). Experimental models of acute pancreatitis and conducted clinical observations indicate a significant share of reactive oxygen species in the pathogenesis. It has

been proved that ethanol induces oxidative stress in the pancreas, however, the molecular mechanism of this process is still unclear.

The metabolism of alcohol leads to the formation of acetaldehyde, which is classified by some researchers as a factor of inducing acute pancreatitis .The first necrotic clinical case of acute pancreatitis in patients after ingestion of 24% acetic acid - oxidized form acetaldehyde was described in 1966 [148]. The authors reported the cause of metabolic acidosis of acute pancreatitis, emphasizing that acetic acid is normal or "safe" biodegradable metabolite of ethanol.

Consumption of ethyl alcohol results in depletion of arachidonic acid (C20:4) AA in membranes of erythrocytes limiting cell plasticity and increase the risk of vascular complications. Peracetic acid in relation to other metabolites of ethanol and peroxides selectively inhibits the acyltransferase responsible for the incorporation of AA acid C20: 4 to erythrocyte membranes in view of the selective oxidation of the thiol residues of the enzyme [149]. Recently demonstrated a significant increase in acetylation of lysine residues of proteins in the liver as a result of treatment with ethanol [150].

One of the consequence of ethanol metabolism in the cells of the pancreas is the growth of vesicular formation of hydrogen peroxide (H_20_2), which reacted with acetic acid can be oxidized to peracetic acid – much higher reactivity. Peracetic acid has the ability to oxidize cysteine, which residues in the protein, while the SIRT3 has the ability to deacetylation of ε-aminoacid lysine residues [151]. In previous studies in rats it was shown that peracetic acid provided at low levels (500nM) directly to the mouth of the hepato – pancreatic gastrointestinal already after one hour caused a significant and marked changes in the appearance of the pancreas of animals. Changes were characteristic of necro-hemorrhagic pancreatitis. Tissue morphology by light microscopy confirmed the inflammation of the organ as a result of supply peroxyacetate, and studies by electron microscopy revealed damage to the structure of mitochondria

(loss of combs), reduced matrix density , and visible swelling endoplasmic reticulum. Also the evidence of increased levels of peroxyacetate tissue changes is more advanced.

Alveolar cells pancreatic cells belong to the most severe protein biosynthesis and digestive enzymes in the human body. Structural integrity of mitochondria in the cells of the vesicular pancreas is essential for the production of ATP, which is necessary for the biosynthesis of proteins in the membrane of the endoplasmic reticulum. Elucidation of the mechanisms of oxidative stress induced by ethanol intoxication can provide some information about new and effective treatment of acute pancreatitis.

1.8. Sirtuine 3 (SIRT3)

Mitochondrial protein hyperacetylation as a result of ethanol consumption has been proposed to play a crucial role in many diseases, ranging from inflammation to cancer [66-69]. We still do not have a wide knowledge about the role of acetylation on noncore histone proteins. Recently it has been shown that about 20% of mitochondrial proteins are regulated through acetylation and other post-translational modifications [70]. Protein acetylation affects many of cellular pathways like: urea cycle, cellular respiration, oxidative stress, lipid metabolism [71-78] and also has been implicated in plenty of diseases like cardiovascular disease, cancer, aging, metabolic disorder and alcoholic liver disease [79-82].

During the deacetylation reaction, sirtuins use NAD^+ as a cofactor resulting in its cleavage to yield nicotinamide (NAM). Consequently, the acetyl group from lysine residues on the substrate protein is transferred to the ADP-

ribose moiety on NAD^+ to yield O-acetyl-ADP-ribose and the deacetylated protein substrate (Fig.9).

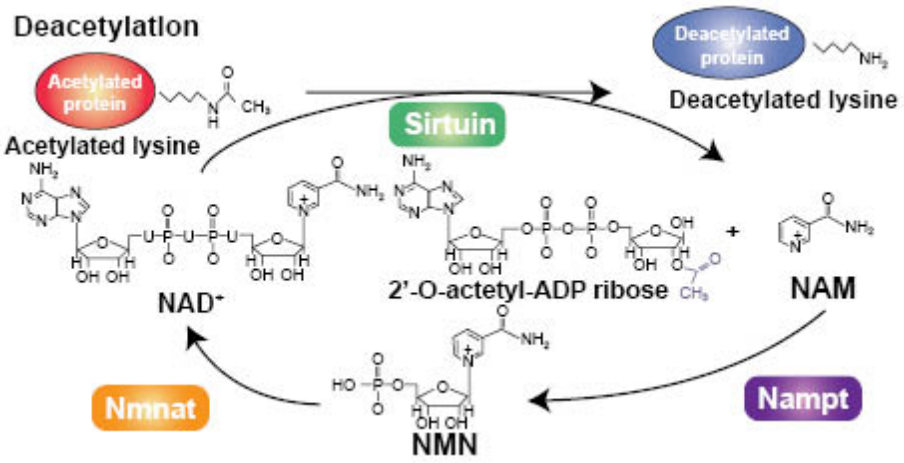

Fig. 9 | The deacetylation reaction of sirtuins [102].

The deacetylation reaction of Sirtuins involves removal of acetyl groups from lysine residues on target proteins thereby deacetylating the protein. This reaction uses NAD as a cofactor which gets converted to O-acetyl-ADP-ribose (O-AADPr) and nicotinamide (NAM).

Protein acetylation occurs on N-ε-lysine residues and has been shown to affect protein structure, function and activity [83-85]. To prevent excessive acetylation of nuclear, cytosolic and mitochondrial proteins cells have evolved a family of proteins known as histone deacetylases (HDACs) [86]. Sirtuins (SIRT) are a family of III class of histone deacetylases and also zinc-requiring, NAD^+-dependent deacetylase enzymes which modulate nuclear, cytosolic and mitochondrial proteins [87]. Currently, the family of sirtuins contains of 7 isoforms. SIRT3, SIRT4 and SIRT5 are situated in mitochondria and regulate most of proteins functions by deacetylation [88].

SIRT3 is a 257 amino acid protein encoded by the SIRT3-gene located on chromosome 11p15.5. The longer, enzymatically-inactive form of SIRT3 is

imported into the mitochondria by the N-terminal targeting sequence, and is 44kDa in length with a 15kDa mitochondrial localization sequence (MLS). While imported to the mitochondria, the MLS is removed by a mitochondrial protein peptidase and the resulting 29kDa, NAD^+-dependent protein that is active and able to deacetylate target proteins. Most researchers suggest that SIRT3 is located only in mitochondria [115,116,117,118], others suggest Sirt3 can also function in the nucleus and relocates to the mitochondria upon stress [117]. Furthermore, reports also suggest that although the long form of SIRT3 (44kDa) can be found in the mitochondria, nucleus and cytoplasm, the short form (28kDa) is exclusively mitochondrial [119]. Both human and mouse SIRT3 contain mitochondrial import signals that are cleaved during its import into mitochondria, giving rise to a shorter active form (Fig.10) [120-122].

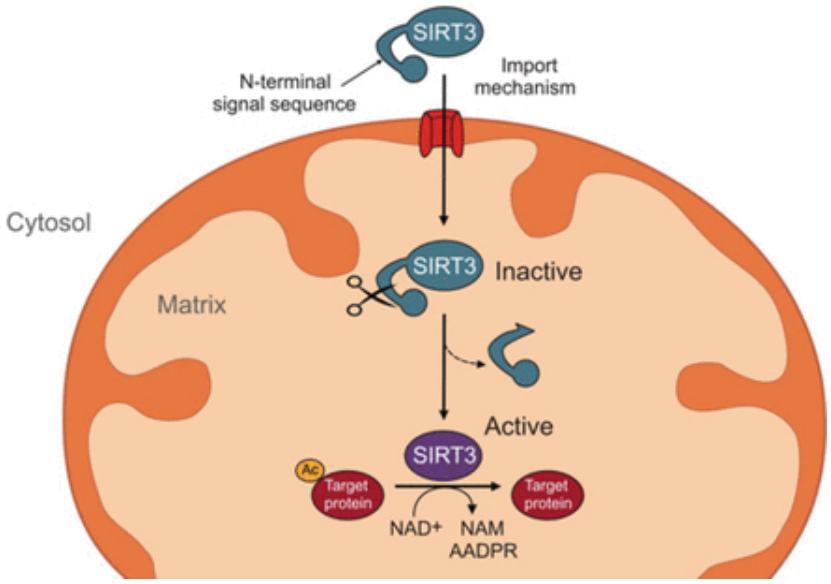

Fig. 10 | SIRT3 is a mitochondrial NAD+-dependent protein deacetylase

SIRT3 is encoded by the nuclear genome and translated in the cytoplasm as a longer enzymatically inactive precursor that is imported into the mitochondrial matrix by a canonical N-terminal targeting sequence. Following import, the targeting sequence is cleaved by a mitochondrial protein peptidase (scissors) leading to the enzymatically active SIRT3.

Recent scientific reports underline the impact of increased acetylation of

mitochondrial proteins on alcohol abuse – dependent diseases in rodent [89,90]. SIRT3 is the main regulating enzyme of mitochondrial proteins acetylation [91]. Moreover, considering that SIRT3 is a NAD^+-dependent enzyme, a significant shift in the balance between NADH and NAD^+ plays a significant role in SIRT3 activity. It has been shown that during chronic ethanol consumption the SIRT3 is downregulated and caused protein acetylation [92-98]. SIRT3 has been also reported as a regulator of antioxidant enzymes such as isocitrate dehydrogenase 2 (IDH2) and superoxide dismutase (SOD2) [99,100].

Various metabolic pathways, that can modulate cell survival and metabolism have critical proteins that are localized in mitochondria and in this respect; SIRT3 has been shown to be a mitochondrial fidelity protein which is important in maintaining mitochondrial integrity and metabolism [101]. Lysine acetylation has recently emerged as an important, and perhaps the primary, posttranslational modification employed to regulate mitochondrial proteins [103,104]. Acetylation of mitochondrial proteins may play a role in maintaining and regulating mitochondrial ROS and superoxide levels as well as function. SIRT3 is a regulatory protein, maintaining mitochondrial homeostasis via changes in the acetylation of metabolic target proteins, including those comprising the mitochondrial ROS detoxification system [105]. Studies on $SIRT3^{-/-}$ mice have shown that SIRT3 protects cells from oxidative stress by activating SOD2, a major detoxification agents of ROS, via deacetylation of two lysine residues, Lys^{53} and Lys^{89}. Another study found that SIRT3 also activates SOD2 by deacetylation of the lysine residue Lys^{122} [109,110]. Thus SIRT3 participates in controlling the levels of intracellular ROS in response to stress via post-transcriptional regulation of SOD2. In addition to ROS detoxification, SIRT3 may directly regulate ROS production. SIRT3 can interact with and deacetylates several components of the mitochondrial electron transport chain, including complex I and III [111,112,113] which are believed to be responsible for 90% of ROS production within mitochondria. The lack of SIRT3 in muscle cells has been shown to reduce mitochondrial oxidation and increase ROS production and oxidative stress [114].

The first SIRT3 substrate identified, as described in two parallel studies published in 2006, was the mitochondrial enzyme AceCS2 (acetyl-CoA synthetase II) [106] that was reported to be activated by SIRT3-mediated deacetylation. AceCSs catalyze the conversion of acetate plus CoA to form acetyl-CoA. In mammals, there are two isoforms of the enzyme: AceCS1, a cytosolic form, and AceCS2, localized to the mitochondrial matrix [107]. Important source of acetate in mammals is the ethanol detoxification pathway. After ethanol intake, acetate levels in human blood increase 20–30 fold [108]. Thus, through AceCS2 activation, SIRT3 could play a role in the metabolism of acetate derived from ethanol.

1.9. Manganese superoxide dismutase (MnSOD)

Superoxide dismutase (SOD) scavenger enzymes are the major ROS detoxifying enzymes protecting the cells from potential damage caused by excessive amounts of ROS (oxidative stress).

Under normal circumstances, the deleterious effects of ROS are avoided by several antioxidant enzyme systems that detoxify ROS produced in the cells [59]. Among the antioxidants, the SOD scavenger enzymes convert superoxide radicals into H_2O_2 and molecular oxygen. There are three SOD enzymes expressed in mammalian cells:

- Copper/zinc-containing superoxide dismutase (Cu/ZnSOD, SOD1) is primarily localized to cytosol [60]
- MnSOD (SOD2) is the mitochondrial antioxidant that exists in the mitochondrial matrix [61,62]

- SOD is the extracellular superoxide dismutase (ECSOD, SOD3) is localized to the extracellular space [63].

Human MnSOD is a tetrameric enzyme with four identical subunits each harboring an Mn^{3+} ion [123]. Human MnSOD gene is located on the 6th chromosome, 6q25.3 region encoding a 223 amino acid (26kDa) precursor monomer containing a mitochondria targeting sequence of 26 amino acids that is required for mitochondrial localization [124]. Human MnSOD resides in the mitochondrial matrix is a highly conserved protein with over 40% sequence homology among human, yeast and E. Coli. Each MnSOD monomer has two distinct domains: an N-terminal helical hairpin domain and a C-terminal a/b domain, containing five alpha helices and three-stranded antiparallel beta sheets. As MnSOD is taken up by the mitochondria, the mitochondria targeting sequence is clipped off, leaving a 22kDa monomer, which later incorporates an Mn^{3+} ion and assembles into an 88kDa homotetramer in the mitochondria. Residues D159, H163, H26, H74, and a water molecule from each subunit contribute to the manganese metal-binding site, namely, the active site [125].

In mammalian cells, manganese superoxide dismutase (MnSOD) is the essential mitochondrial antioxidant enzyme that detoxifies the free radical superoxide, the major by-product of mitochondrial respiration [57]. The crucial role of MnSOD is protecting cells against oxidative stress [58], maintaining cellular physiology in response to genotoxic conditions, such as oxidative stress. In addition to MnSOD gene transcription, MnSOD enzymatic activity is enhanced via post-translational modifications.

Post-translational modifications of proteins control many biological processes through a variety of mechanisms that include the changes in protein activity, interactions and subcellular localizations. Those modifications are: phosphorylation, methylation, acetylation, nitration and gluthathionylation.

Although the exact mechanisms associated with the overexpression and/or activation of MnSOD are not fully understood, there are a great deal of reports exploring the role of post-translational as well as transcriptional and post-transcriptional regulation of MnSOD in governing its superoxide dismutase activity in the adaptive response against oxidative stress. MnSOD acetylation represents an alternative mechanism of post-translational regulation of MnSOD, which may contribute to the overall MnSOD activity in stress response. The discovery of the mitochondrial sirtuin family – NAD^+-dependent protein deacetylase enzymes (SIRT3, SIRT4 and SIRT5) it was reasonable to suggest the existence of substrates to deacetylation reaction inside the mitochondria [64]. Additional support came from the studies observing significantly increased MnSOD acetylation and corresponding decrease in the MnSOD superoxide dismutase activity in SIRT3-knockout cells [110]. Tao et al. further identified Lys^{122} of MnSOD as a reversibly acetylated residue [126]. These pioneering investigations on the regulation of MnSOD activity by changes in specific lysine acetylation are followed by the reports on SIRT3-mediated regulation of MnSOD activity. Qiu and collaborators found that calorie restriction induces SIRT3-mediated MnSOD activity resulting in reduced oxidative stress. In the same study, Lys^{53} and Lys^{89} were identified as acetylated residues that control enzymatic function [127].

1.10. Heat shock protein (Hsp60)

Heat shock proteins family is generally responsible for preventing damage to proteins in response to high levels of heat. They are classified into six major families based on their molecular mass: small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP110 [128]. Hsps are widely represented among prokaryotes and eukaryotes group of chaperones. Representatives sHsps characterized as small heat shock protein homology (exceeding 20%) and similar features of these proteins are low molecular weight (15-40kDa), create hetero- or homooligomeric forms. sHsps participate in protection against irreversible denaturation of the polypeptides by bounding substrates and keeping them competent to folding. Because they have no ATPase activity, in the process of restoring the native conformation of denatured proteins interact with other Hsp. Thus, an important part of an extensive network of chaperones and protease, whose mission is to "control the quality of process of folding of newly synthesized proteins, renaturing denatured polypeptides under conditions of stress or degradation. sHsps are considered the first line of defense cells against the effects of stress, because they do not require ATP substrates bind with high efficiency and their level after the shock increases significantly. Interest in small heat shock proteins increased when it was reported that, outside the stressful conditions, as typical chaperones they are involved in numerous physiological processes. In mammalian cells they are involved in regulation of apoptosis, the process of malignant transformation, and cytoskeletal cells differentiation. Mutations in the human sHps lead to diseases such as cataracts, desminopatia or neuropathy and it is also found that the sHsps part of protein aggregates formed in the course of diseases such as cystic fibrosis, multiple sclerosis or diseases of a neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington) [154].

Heat shock protein 60 (Hsp60) is a chaperonin, located in mitochondria, which may facilitate the right folding of polypeptide chains imported from cytoplasm to mitochondrial matrix. HSP60 functions as a chaperonin to assist in folding linear amino acid chains into their respective three-dimensional structure.

HSP60 monomers form a complex arrangement as two stacks of 7 monomers each. This complex binds to unfolded proteins and catalyze their folding in ATP dependent manner.

Hsp60 is a phosphoprotein whose expression and function can be further modulated by ubiquitination, sumoylation, acetylation, malonylation, N-glycosylation or O-GlcNAcylation [129-135]. Evidence for an acetylation of Hsp60 has emerged after the discovery of the histone deacetylase 1 (HDAC-1) as being an interaction partner of Hsp60 [136]. Mass spectrometric analysis identified several lysyl residues as acceptor sites for acetyl residues within Hsp60 [137].

Hsp60 is constitutively expressed under normal conditions and induced by different types of stressors such as oxidative stress [138,139]. Hsp60 has been considered a major defense system against cellular damage after ethanol administration in the liver and pancreas [140]. Over the past decade investigators found that HSP60 and the pancreatic enzymes share a common location inside the pancreatic acinar cells, interacting intimately [141,142]. HSP60 showed an increasing gradient of collocation along the pancreatic secretory pathway from the rough endoplasmic reticulum and Golgi apparatus to zymogen granules in the acinar cells [141]. An increased transcription and production of HSP60 as protective agent has been proposed in pancreatitis [143-145]. It has been shown that HSP60 plays an important role in the protection of pancreatic tissues against damages and malfunctioning of HSP60 effect under physiological conditions is responsible for the early zymogen activation in AP [146].

The discovery by Allfrey and his co-workers [147] of histone acetylation at the ε- amino group of conserved lysine residues opened a period of almost half a century of studies on the function of this reversible modification in regulating gene expression. Histone acetylation has been correlated with transcriptional activation by creating a more relaxed opened structure of the chromatin and thereby allowing the access of transcriptional machinery to DNA, but also by enhancing the recruitment of transcription factors needed for activation of gene expression [147]. It has been shown that hyperacetylation of Hsp60, as a result of SIRT3-knockdown, resulted in increasing of oxidative stress level.

1.11. Peracetic acid (PAA)

A peroxy acid is an acid that contains an acidic -OOOH group. Peracids are general oxidants, usually with electrophilic properties. The electron-withdrawing character of the substituents determines the reactivity of the peracid. If the parent acid is stronger, the derived peracid is more reactive. The relatively weak oxygen-oxygen linkage (bond dissociation energy of 20 to 50 kcal $mole^{-1}$) is characteristic of organic and inorganic peroxide molecules. Essentially all of the features of peroxide reactivity are associated with the tendency for spontaneous change to form more stable products. The unusual weakness of the –O-O- bond is probably a consequence of the molecular and electronic structure of peroxide molecules and of the relatively high electronegative character of the oxygen atoms [159].

Peracetic acid is probably the most toxic (chronic and acute) of the percarboxylic acids. The acute toxicity of peracetic acid is relatively low [oral LD_{50} (rat) 1540 mg/kg; dermal LD_{50} (rabbit) 1410 mg/kg]. It is highly irritating to skin, eyes and mucous membranes and may be weak carcinogen in mice. There is no such evidence to suggest carcinogenic, reproductive or developmental toxicity in humans. The mechanism of action of PAA is thought to function as other oxidizing agents, i.e., it denatures proteins, disrupts cell wall permeability, and oxidizes sulfhydral and disulfide bonds in proteins, enzymes, and other metabolites [160].

2. SPECIFIC AIM AND HYPOTHESIS

The aim of this thesis is to investigate the molecular mechanisms of induction of acute pancreatitis by peracetic acid as the potent end product of alcohol metabolism. SIRT3 is NAD^+-dependent deacetylase containing in position 280 - redox active cysteine. Oxidative modification of Cys^{280} results in inactivation of this mitochondrial enzyme. The selective oxidation of the thiol residues of the proteins by percarboxylic group of the peracetic acid can oxidize residue Cys^{280} to the sulfonic acid – that can lead to inactivation of SIRT3. Such defined prooxidative impact of peracetic acid may in turn result in mitochondrial proteins, Hsp60 and MnSOD hyperacetylation, which leads to an increase of the superoxide level causing oxidative stress. The defined effects of peracetic acid prooxidative effect will explain the molecular mechanisms of damage to mitochondrial structure and function of the potential ethanol metabolite: peracetic acid.

In this study I wanted to examine if well-known chemistry reaction (Fig.10) can occur also *in vivo*? If this simple chemical reaction with the participation of physiologically occurring hydrogen peroxide and the well-known metabolite of alcohol - acetic acid, actually leads to the formation of highly reactive peracetic acid, it can completely change the insight into the pathogenesis of acute pancreatitis.

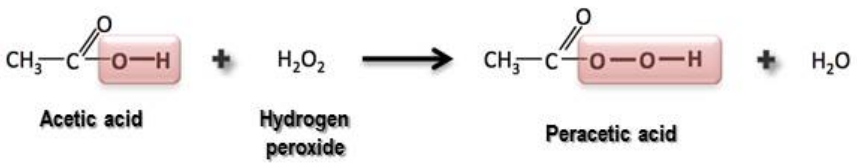

Fig.11 | Reaction of peracetic acid formation.

The expected result of the impact of peracetic acid in the cells in the pancreas is an increase of oxidative stress due to inactivation of SIRT3, as the consequence of mitochondrial superoxide dismutase (SOD2) and heat shock protein Hsp60 acetylation. Hyperacetylation of SOD2 restricts the superoxide anion access to the active site of the enzyme, which will result in increased oxidative stress. Hyperacetylated Hsp60 protein inactivates the thermal pathway and it is responsible for protecting the structure of mitochondrial proteins. The whole hypothesis is presented on diagram below (Fig.12).

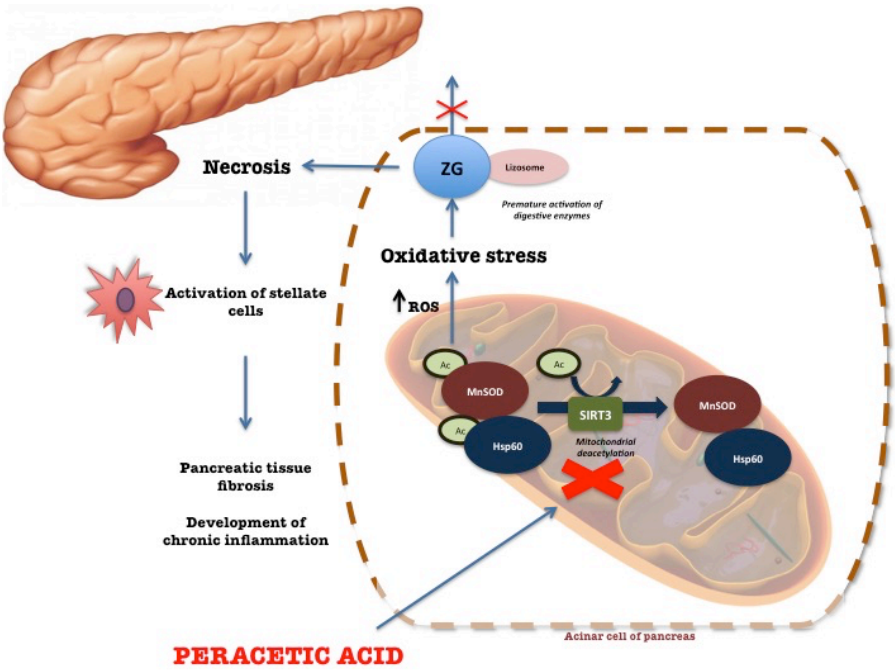

Fig.12 | Diagram presents the hypothesis of my research

Starting in mitochondria, SIRT3 is inactivated by peracetic acid, what in turn results in acetylation of mitochondrial proteins like MnSOD and HSP60. It leads to oxidative stress induction that is involved in premature activation of zymogen granules. The cascade of digestive enzymes activation leads to necroinflammatory response of pancreas, necrosis of organ tissue and activation of stellate cells, what in turn ended up with tissue fibrosis.

3. MATERIALS AND METHODS

3.1. Materials

Reagents	Company
Cell culture medium Ham's F-12K, trypsin-EDTA solution, penicilin/streptomycin solution	**Gibco**
Peracetic acid, Acetic acid, Hydrogen peroxide, dimethyl sulfoxide (DMSO), fetal bovine serum, antibiotic coctail, dexamethasone, mouse monoclonal anti-HSP60, Clone LK1	**Sigma-Aldrich, Poland**
RIPA buffer , Halt Protease/Phosphatase Inhibitor Single-Use Cocktail	**Thermo Scientific**
mouse monoclonal SOD2/ MnSOD antibody	**Abcam**
Protein A– agarose beads , horseradish peroxidase-conjugated antibodies against beta-actin	**Santa Cruz Biotechnology**
nitrocellulose membranes, ECL chemiluminescence reaction	**Amersham**
acetylated lysine primary antibodies	**Cell Signalling**
Annexin V/ PI assay	**BD Pharmigen**
2',7'-dichlorodihydrofluorescein diacetate	**Invitrogen**

3.2. Cell culture

For a cellular model of acute pancreatitis I chose rat pancreatic exocrine, acinar cells obtained from American Tissue Type Collection.

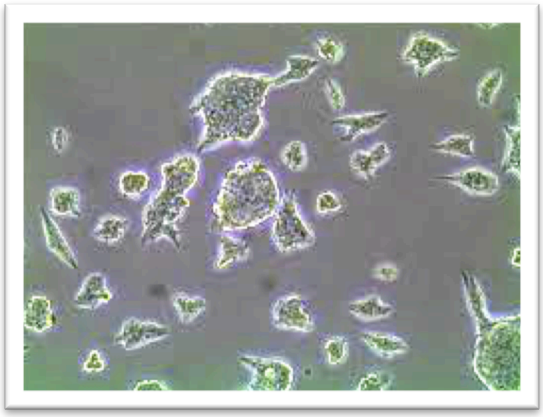

Fig.13 | AR42J cell line (155)

AR42J cells (rat pancreatoma, ATCC CRL1492) was purchased from American Type Culture Collection and maintained in Ham's F-12K (Gibco) medium supplemented with 20 % fetal bovine serum, 100 µg/mL penicillin/streptomycin and 2mM L-glutamine (all the supplements were obtained from Sigma Aldrich). Cells were cultured in humified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were cultured in plastic tissue flasks (T-25, T-75) with vented caps and stored in an incubator. Cells were passaged every 2-3 days (in 1:3 ratio) in order to avoid confluence of the cultures. To subculture adherence cells for growth, medium was aspirated and cells were washed in 10 ml of pre-warmed PBS, 1 ml trypsin-EDTA was added and placed in an incubator for 3 minutes at 37°C. Following trypsinization, 10 ml of complete medium was mixed with the detached cells and 2.5 ml aliquot of the cell

suspension was transferred to a new flask containing 12.5 ml of complete F-14 K medium. Cells were left overnight in the incubator to adhere to the culture flask. Cells were seeded into 60 mm cell culture plates for immunoblotting, electron microscopy and immunoprecipitation, 6-well culture plates for flow cytometry, and determination of the generation of reactive oxygen species (ROS) and analysis of apoptosis, 96-well plates for MTT assay. All the experiments was performed under microbiological safety cabinet.

3.3. Cell treatment

Before each experiment cells were trypsinized with a solution of 0,25% trypsin and 0,02% EDTA and seeded onto 96-well, 6-well plates, 60mm cell culture dishes or T-75 and T-25 flasks dependent on planned experiment. After one day (when the cells are attached) medium was replaced with the one containing dexamethasone (DEX) – 1μM DEX phosphate (Sigma-Aldrich, Poland) for seven days, to develop acinar cell-like phenotype. It has been shown that dexamethasone treatment of AR42J cells induced expression of pancreatic exocrine markers [152]. DEX converts them into mature exocrine cells secreting digestive enzymes, inducing phenotype changes like secretory organelles (zymogen granules) increase [153].

Fig. 14 | After each pretreatment with 1µM DEX, the cells were ready for further investigations (155).

After pretreatment the cells were ready for further experiments. Medium was changed into the one without dexamethasone but containing proposed ethanol metabolites: peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and mixture of AA with H_2O_2 in different concentrations respectively and treated for 24h. After the proper incubation time the cells were harvested or assayed.

3.4. Cell lysates preparation

To run a western blot the cells have to be lysed in order to release the proteins of interest. AR42J cells were scraped into PBS, washed twice with cold PBS and added 350µl of lysis buffer per well containing 1ml RIPA buffer (Thermo Scientific) with 10µl protease inhibitors and 10µl phosphatase inhibitors (Halt Protease/Phosphatase Inhibitor Single-Use Cocktail (100x) (Thermo Scientific) and 20µl complete EDTA-free protease inhibitor cocktail. The cells were freeze-thawed 3X and cell debris removed by centrifugation (12 000 x g, 15 minutes at

4℃) and the supernatant stored at -80℃. The pellet was resuspended in an equal volume of lysing solution and represented the membrane fraction. Protein concentrations were calculated using the Bradford calorimetric method. It is based on the binding of Coomassie Brilliant Blue G-250 dye to the proteins and particularly basic and aromatic amino acids residues. Under acidic conditions, the dye is predominantly in the protonated cationic form (red colour). When the dye binds to proteins, it is converted to a stable unprotonated form (blue color). It is this blue unprotonated form that is detected at 595nm to quantify the concentration of proteins. The standards and samples were set up in triplicate, 10µl per well into 96-well plate. 190µl filtered 1 in 5 dilution of Bradford's assay solution was supplemented to each well. The assay color developed instantly and was read on plate reader at 595nm. Protein concentrations were calculated according to standard curves.

3.5. Immunoprecipitation

For immunoprecipitation, 5µg of primary antibodies: mouse monoclonal anti-HSP60, Clone LK1 (Sigma-Aldrich) and mouse monoclonal SOD2/MnSOD antibody (Abcam) was added per 1ml of total cell lysate. Than they were incubated for 16h at 4°C with gentle rotation. Protein A–agarose beads (Santa Cruz Biotechnology) were added to the samples and incubated for an additional 2h in 4°C to immunoprecipitate antibody/protein complex. Nonspecifically bound proteins were removed by repeated washing with isotonic lysis buffer. For Western blot analysis, immunoprecipitated proteins were run on a 10% SDS gel. Proteins were next transferred to nitrocellulose membranes (Amersham, Les

Ulis, France), subsequently preincubated for 1h in blocking solution (3% BSA, 0,1% Tween-20 in PBS) and incubated using acetylated lysine primary antibodies (Cell Signaling, Italy). The membranes were then washed in PBS, containing 0,1% Tween-20, and reincubated with a 1:2000 dilution of horseradish-conjugated anti-rabbit IgG antibodies. Immunoreactivities were revealed using the ECL chemiluminescence reaction (Amersham). Each experiment was performed at least three times.

The protein bands observed on films were quantified by using Image J software. Three readings of each band were measured and then each band was normalized to its correspondent band with β-actin. After that, the average of the three readings of each experiments were measured and presented as chart bars in Excel (Microsoft). The final average and standard deviation readings were measured by taking the values of three independent experiments.

3.6. Cell viability assay using MTT

The MTT-cell proliferation assay is a quantitative colorimetric assay for measurements of cellular proliferation, viability and cytotoxicity [156,157]. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity [158]. The mitochondrial activity of the cells is based on conversion of the MTT (tetrazolium salt) dye to a purple colored formazan crystal by the active mitochondrial reductases present in the viable. The purple color thus formed is directly proportional to the viable cells present.

MTT assay was used to detect the viability of AR42J rat pancreatic exocrine cells after treatment with ethanol metabolites. AR42J cells were seeded onto 96-well plates at a density of 3000 cells per well and cultured for 24h. Medium was then removed and replaced with the one containing dexamethasone added freshly on the same day. Medium with dexamethasone was changed daily, and the cell growth was inhibited by the action of glucocorticoid, which did not allow the overgrowth of cells monolayer during the seven days of pretreatment.

After pretreatment with dexamethasone, cells were treated with serial dilutions of peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and acetic acid combined with hydrogen peroxide within the range of 15,625 μM–500 μM. The incubation time was 24h. Then, cells were suspended in solution of 0.5 mg/mL MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and incubated at 37°C for 4h, till the purple precipitate (formazan) was clearly visible under the microscope. The supernatant was removed. Finally, 100μL of DMSO was added and the plates were protected from light, and shaken for 30 minutes on a rotating platform to dissolve the precipitate of formazan – product of MTT reduction by metabolically active cells. Absorbance at 570nm was determined using a microplate reader Jupiter (Biogenet) using DigiRead Communication Software (Asys HiTech GmbH). The number of cells was calculated based on absorbance values. The results were presented as a percentage of control. Each experiment was performed at least three times and obtained data was presented and analyzed using GraphPar Prism Software (GraphPad Prism v.5).

3.7. Flow cytometry analysis of apoptosis

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry that distinguishes single cell in a population and sorts them into different categories by evaluating specific light scattering and fluorescence emission characteristics from cells. Cell suspension was hydrodynamically manipulated into a single cell alignment by sheath fluid as cells are intersecting across the argon ion laser. Lasers excite fluorescent dyes by sending out monochromatic light of specific wavelengths, which are deflected or reflected depending on the size and density of the cell. The light emission from the dye is assayed by photon detectors, and computer software can analyze the data.

The externalization of phosphatidylserine is a well-recognized event occurring in cells undergoing apoptotic cell death. Binding of fluorescein isothiocyanat (FITC) labeled Annexin-V is commonly used to detect phosphatidylserine residues present on the outer cellular membrane of apoptotic cells. Necrotic cell death in turn can be visualized by propidium iodide, a dye, which upon binding to DNA shows a pronounced shift in absorption characteristics. Under physiological conditions cellular membranes are impermeable for propidium iodide, resulting in no fluorescent staining of nuclei. Membrane leakage, occurring in necrotic cells, however, significantly increases the cellular permeability for propidium iodide, thus staining nuclei of cells prone to undergo necrosis. Plots from gated cells illustrated the populations corresponding to viable (Annexin V^-PI^-) cells, apoptotic (Annexin V^+PI^-) cells, apoptotic/necrotic (Annexin V^+PI^+) cells and to dead (Annexin V^-PI^+) cells.

The AR42J cells were seeded onto 6-well plates, 300 000 cells per well. After 7 days of pretreatment the medium was changed into the one containing appropriate concentrations of peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and acetic acid combined with hydrogen peroxide. After

incubation, cells were pelleted and incubated with Annexin V and PI according to manufacturer's protocol. Finally, the cells were analyzed using BD FACScan detecting the cell fluorescence intensity, using channel FL1 and FL2. The experiment was repeated three times. The results were then analyzed using CellQuest Pro Software.

3.8. Evaluation of reactive oxygen species (ROS) generation

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a chemically reduced form of fluorescein used as a molecular probe for detecting intracellular ROS levels. H2DCFDA is permeable across cell membrane in cells. The DCFDA uptake by the cells will be deacetylated by esterases forming 2', 7'dichlorofluorescein (DCFH), a non-fluorescent intermediate product with impaired cell membrane permeability, and trapped within cells. Upon oxidation by intracellular ROS molecules, DCFH were oxidized into the fluorescent product 2',7'-dichlorofluorescin (DCF) .

AR42J cells were seeded onto 6-well plates at a density of 300 000 cells per well and cultured for 24h. The following day, the medium was replaced with the one with 1μM dexamethasone, and changed daily during 7 days of pretreatment. After seven days the AR42J cells were treated with 50μM and 500μM of peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and acetic acid combined with hydrogen peroxide for 24h. Finally, harvested using Trypsin-EDTA solution. Cells were transferred into 15ml centrifuge tubes and centrifuged at 1,200 x rpm for 3 minutes. Cell pellet was washed with PBS and

submitted to incubation with H_2DCFDA (Invitrogen, UK). 100µL of 100µM H_2DCFD was added to each well, and incubated in the dark at 37°C for 30 minutes. After incubation, cell samples were washed 3 times with PBS and resuspended in 500µl of PBS. The samples were then subjected to FACS analysis using FACs calibur from BD Biosciences. The experiment was repeated three times. The results were then analyzed using CellQuest Pro Software.

3.9. SIRT3 activity

To examine Sirtuine-3 activity after treatment with different concentrations of potential alcohol metabolites SIRT3 Direct Fluorescent Screening Assay Kit (Cayman Chemical Company cat. no.10011566) was used. It was performed according to manufacturer protocol. Briefly, the procedure requires two easy steps, both performed in the same microplate. In the first step, the substrate, which comprises the p53 sequence Gln-Pro-Lys-Lys (ε-acetyl)-AMC, is incubated with human recombinant SIRT3 along with its co-substrate NAD^+. Deacetylation sensitizes the substrate such that treatment with the 50µM and 500µM solutions of peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and acetic acid combined with hydrogen peroxide in the second step releases a fluorescent product. The fluorophore was than analyzed with an excitation wavelength of 350-360nm and an emission wavelength of 450-465nm.

3.10. Electron microscopy

Before experiments cells were seeded onto 60mm culture dishes Detached and adherent cells growing on the 60mm culture dishes were fixed in situ by the direct addition of an equal volume of 8% glutaraldehyde in 0.2M cacodylate buffer (pH 7.4) to the dish right after 24-hours treatment. After fixation for 1h at 43°C, attached cells were scraped off and the whole suspension was centrifuged at 10 000 x g for 5min., washed in 0.1M cacodylate buffer (pH 7.4), post-fixed with 1% osmium tetroxide for 1h. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (JEM 1200 EX II, JEOL Ltd., Japan).

The experiments with TEM was obtained thank to help of Prof. Zbigniew Kmieć, and Dr Agata Zauszkiewicz-Pawlak from Histology Department of Medical University of Gdansk, Poland.

3.11. Statistical Analysis

Results are expressed as mean ±SD from at least three independent experiments. Significance was determined by an unpaired 2-tailed Student's t-test or by two-way ANOVA with statistical significance set at $P<0.05$. As indicated in each figure *$P<0.05$, **$P<0.01$. Data was analyzed using GraphPad Prism (GraphPad Prism software, La Jolla, CA).

4. RESULTS

4.1. MTT assay

In order to determine the influence of 24h treatment of AR42J cells with potential ethanol metabolites on cell viability MTT assay was performed. The cells were treated for 24h with serial 2-fold dilutions of peracetic acid (PA), acetic acid (AA), hydrogen peroxide (H_2O_2) and acetic acid combined with hydrogen peroxide (equimolar concentrations) within the range of 15,625μM – 500μM. After the appropriate incubation time 0,5mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added.

Peracetic acid (PA), hydrogen peroxide (H_2O_2) and mixture of both: PA and H_2O_2, shows an inhibitory effect on AR42J cells viability in concentration dependent manner. All of them inhibited cell viability significantly about 250μM and 500μM concentration. Both PA and H_2O_2 in 500μM concentration decreased the cell viability from 100% to 44,9% and 57,8% respectively, as compared to control. AA shows no significant effect on AR42J cell viability. The results of MTT assay was used in subsequent investigations – I choose to examine two boundary concentrations of ethanol metabolites: 50μM and 500μM. According to results presented below, the first one should show no significant changes, while the second should have the opposite, prooxidative and destructive effect to the acinar cells of the pancreas.

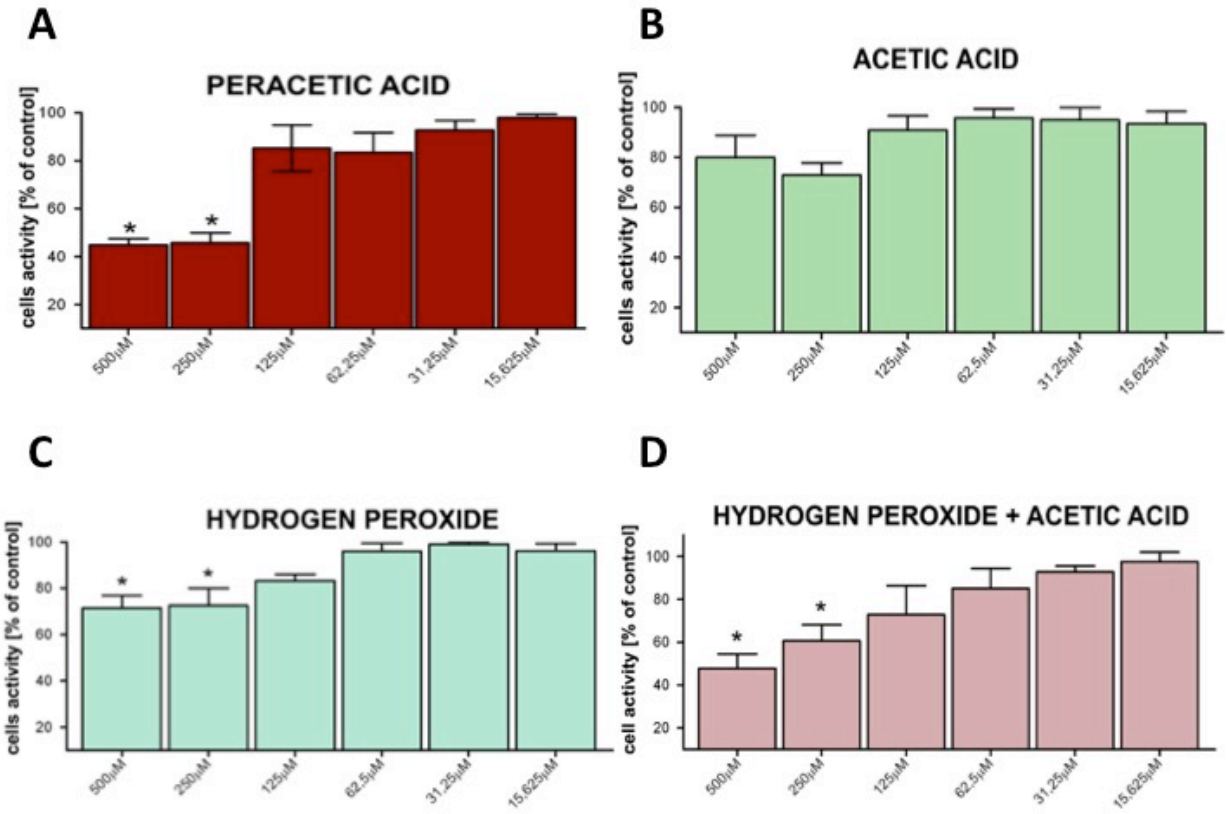

Fig.15 | Ethanol metabolites inhibits AR42J cell growth.

Cells were treated with serial dilutions of peracetic acid (A), acetic acid (B), hydrogen peroxide (C) and acetic acid combined with hydrogen peroxide in equimolar concentrations (D) within the range of 15,625µM – 500µM. The incubation time was 24h. The cell viability was then determined by means of MTT assay. The results were presented as a percentage of control. Data from at least three independent experiments are presented as mean ± SE. Data were analyzed by GraphPad Prism Software version 5.01 performing One-way ANOVA combined with Tukey's Test. *P<0.05 vs. control

It has been shown in Figure 16, that treatment with at least 250μM (or more concentrated) PA, H_2O_2 or mixture of H_2O_2 and AA decreased significantly AR42J cells viability, while the same concentrations of AA shows no influence.

4.2. Flow cytometry analysis of apoptosis

To measure the effects of potential ethanol metabolites on cell death induction; cells were harvested by trypsinisation, stained with propidium iodide (PI) and Annexin V-FITC for flow cytometer analyzed at 15 000 events. AR42J cells that were necrotic did pick propidium iodide and were located at the upper left part of the flow cytometer chart while those that were at their early, late and did show apoptosis will be located at lower right and upper right. From flow cytometer observations, there was about less than 1 % apoptosis in cells that were not treated with any ethanol potential metabolite examined in this study. In cell treated with both 50μM and 500μM PA, AA, H_2O_2, and AA+ H_2O_2 there was a shift towards apoptosis with about 10% in PA, AA and AA+ H_2O_2 treatment. However H_2O_2 in both concentrations presents the increase of apoptotic and necrotic cells (Fig.16). From both flow cytometer and cell cytotoxicity assays, the following 50μM and 500μM concentrations were chosen as appropriate for further studies.

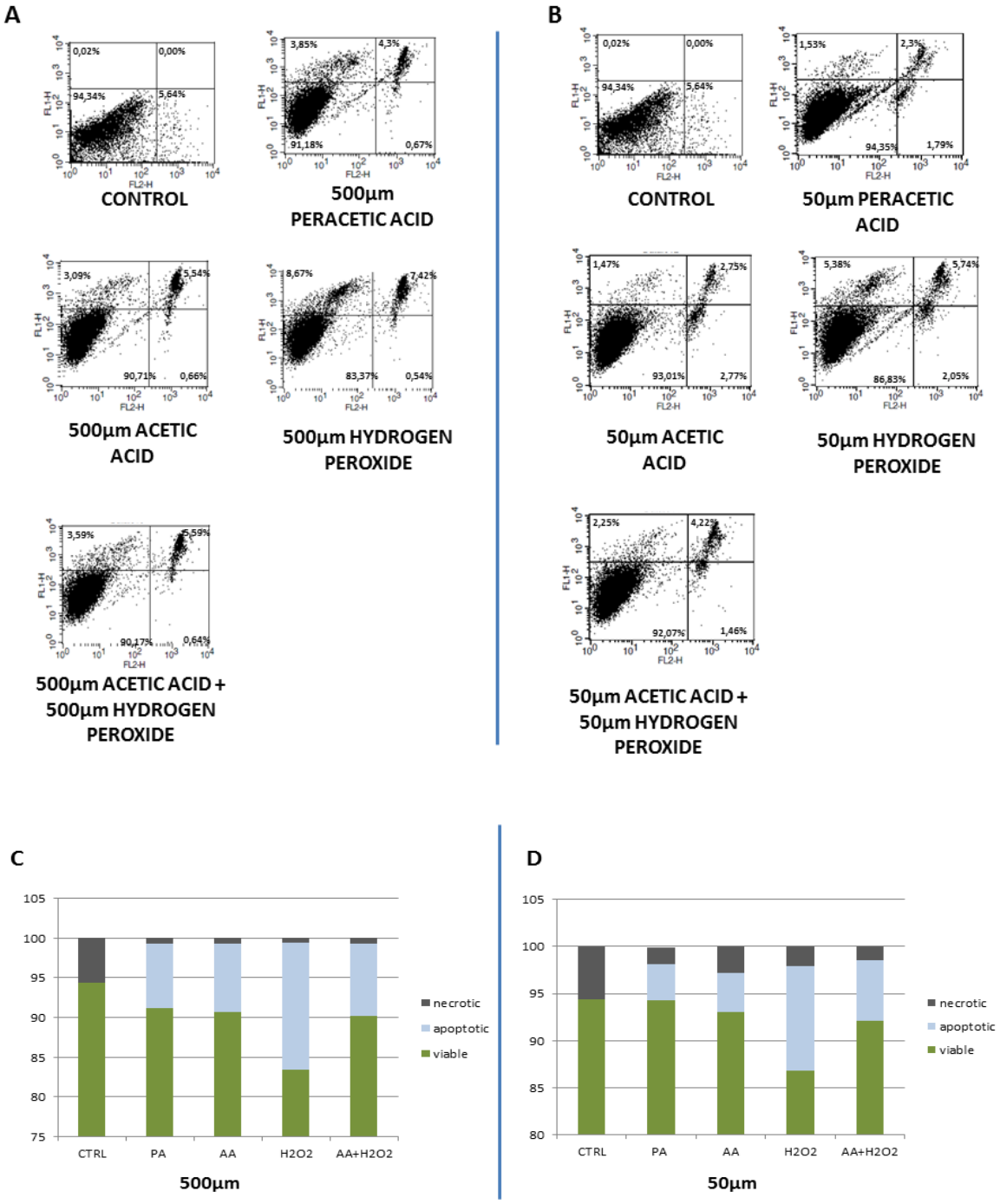

Fig. 16 | Induction of AR42J cell death after treatment with 50µM and 500µM PA, AA, H_2O_2, and AA+H_2O_2 mixture for 24h.

The cells were harvested and the percentage of apoptotic and necrotic cells was determined performing double staining with PI-Annexin V-FITC. 50µM concentration of all investigated compounds, presented significant increase of both apoptotic and necrotic cells (A). 500µM concentrations shows apoptotic/necrotic cells in all treatments as well as 50 µM (B). Plots are representative of three independent experiments. The incubation time was 24h.The diagrams in C and D presents the changes after treatment with 50µM (C) and 500µM (D) concentrations of examined compounds.

4.3. Detection of ROS generation

In order to investigate the level of ROS generation after treatment with different concentrations of PA, AA, H_2O_2, and AA+ H_2O_2, samples were prepared as described in Materials and Methods and analyzed using BD FACScan cytometer.

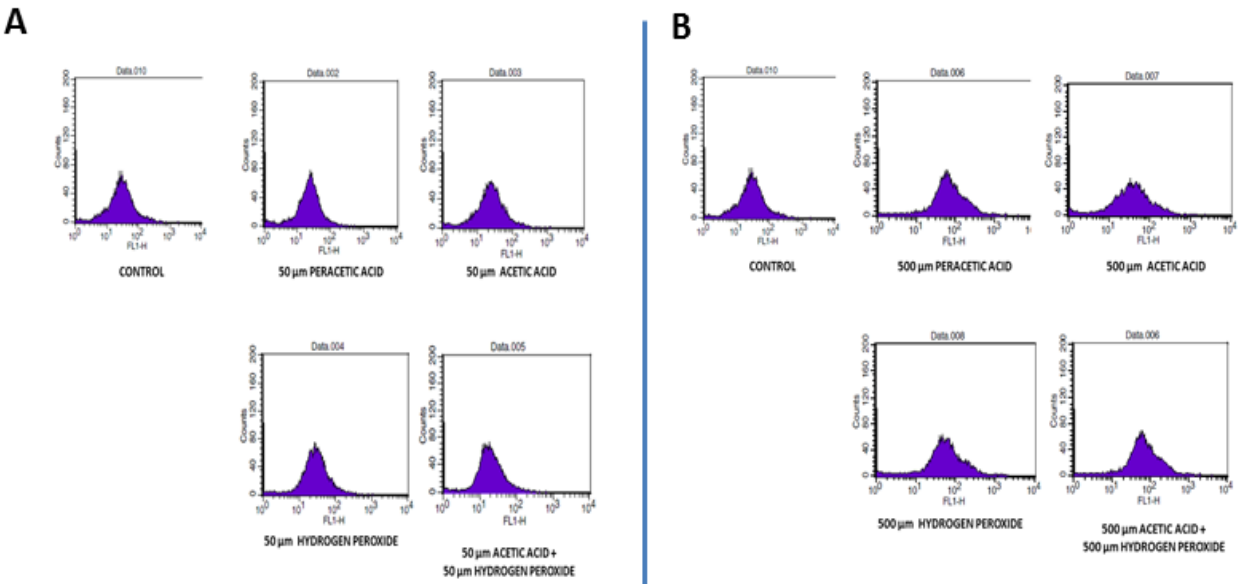

Fig. 17 | Detection of ROS generation carboxyl-H2DCFDA dye after treatment with 50µM PA, AA, H_2O_2, and AA+ H_2O_2

ROS induces the modification of carboxy-H2DCFDA that fluoresces green as detected by flow cytometry. The fluorescent peak of treated cells shift compared to the peaks in controls shows no changes in all treatments, suggest low level of ROS generation according to 50µM concentration of examined compounds (A), 500 µM PA and H_2O_2+AA treatment shows significant shift suggesting ROS generation increase (B). The incubation time was 24h.

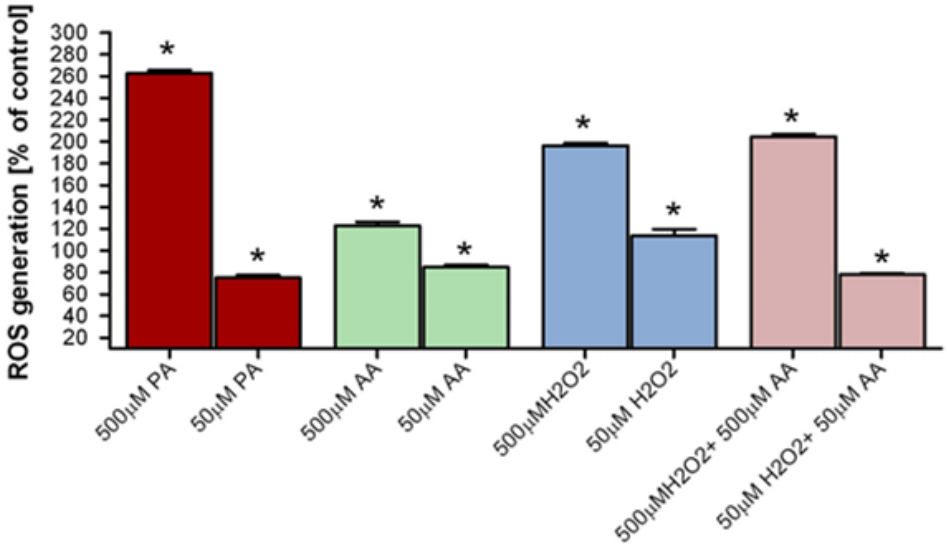

Fig. 18 | Detection of ROS generation carboxyl-H2DCFDA dye after treatment with 50µM PA, AA, H_2O_2, and AA+ H_2O_2

Statistical analysis of obtained results suggested that treatment with both concentrations of all examined compounds lead to statistically significant increase of reactive oxygen species level. The incubation time was 24h.

Obtained results shows that even 50µM compounds treatment significantly changes ROS level, but the fold of change after 500µM PA, 500µM H_2O_2, 500µM mixture of AA and H_2O_2 treatment reveal much higher growth of ROS level than acetic acid on his own. These results provide a promising basis for further discussion of the established hypothesis, according to which peracetic acid induces oxidative stress and underlies the pathogenesis of acute pancreatitis by inactivation of SIRT3, increasing ROS and prematurely activation of proenzymes.

4.4. Immunoprecipitation

In order to obtain the influence of ethanol metabolites on mitochondrial proteins acetylation a double-labeled immunoprecipitation of acetylated lysine and Hsp60/MnSOD was performed. Results revealed that treatment of AR42J pancreatic acinar cells by 50μM concentration of PA and mixture of 50μM H_2O_2 and 50μM AA increase post-translational modification of the protein HSP60. Significantly expanded Ac-Hsp60 level was observed as well in AA and H_2O_2, but the fold of change was not that evident as in peracetic acid (Fig.19A).

The influence of all investigated compound on MnSOD protein acetylation was clear - they all increase the level of MnSOD acetylation significantly as shown in Fig.19B

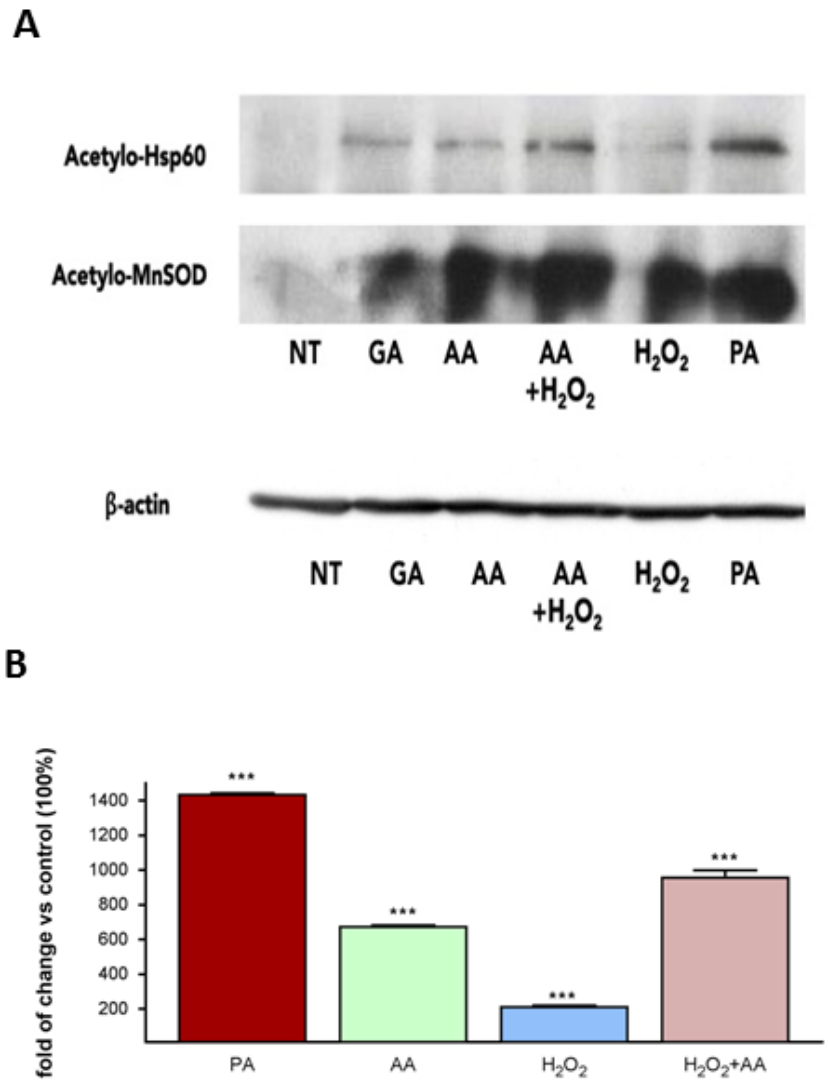

Fig. 19 | Mitochondrial proteins acetylation is induced by ethanol potential metabolites.

Semiconfluent cells were treated with 50µM acetic acid, acetic acid combined with hydrogen peroxide, hydrogen peroxide and peracetic acid for 24h, lysed and immunprecipitated using anti-MnSOD and anti-HSP60 antibodies. The immunoprecipitates were separated by a 10% SDS–PAGE, blotted onto a nitrocellulose membrane, incubated with an anti-acetylated lysine antibody and revealed using the ECL system as described in Materials and Methods (A). Densitometry carried Using Image has demonstrated the change in the level of acetylation of the protein Hsp60 disclosed statistical significance (B).

4.5. SIRT3 activity

To examine SIRT3 activity SIRT3 Direct Fluorescent Screening Assay Kit (Cayman Chemical Company cat. no.10011566) was used. It was performed according to manufacturer protocole. The results revealed that 500μM solutions of peracetic acid, acetic acid and mixture hydrogen peroxide and acetic acid (resulting in peracetic acid formation) has an inhibitory effect on SIRT-3 activity, and for all of those compounds the inhibition level exceeded 100%. 50μM concentration of examined compounds also shows an significant inhibitory effect (about 40% in comparison to control).

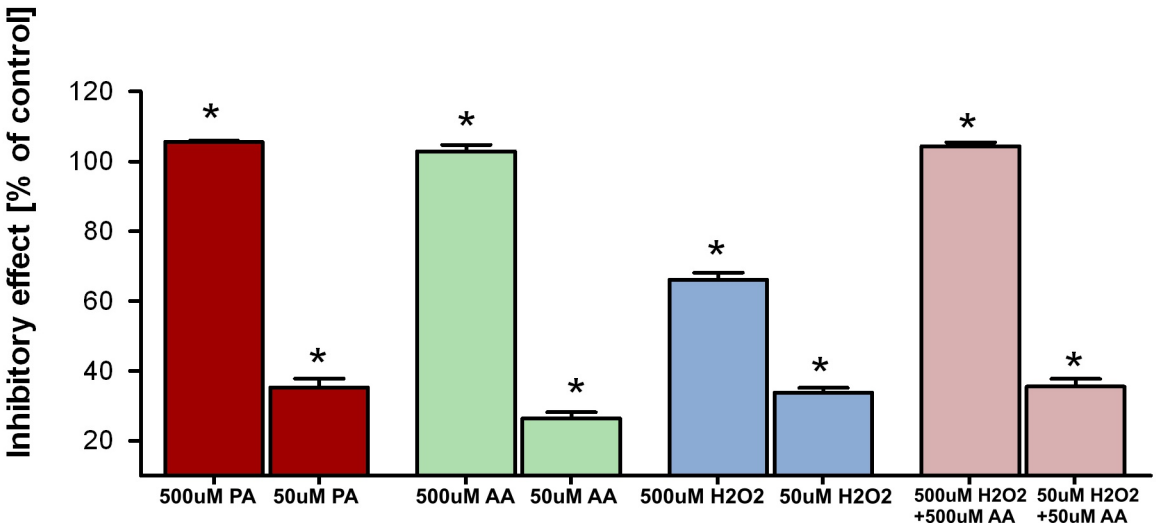

Fig.20 | Inhibitory effect of potential ethanol metabolites on SIRT3 activity

Data from at least three independent experiments are presented as mean ± SE. Data were analyzed by GraphPad Prism Software version 5.01 performing One-way ANOVA combined with Tukey's Test. *P<0.05 vs. control

4.6. Electron microscopy

In order to achieve an eye evidence of harm caused by ethanol potential metabolites tested in this research was performed as previously AR42J cells were treated with 50 and 500μM concentrations of test compounds and phenotypic changes were observed by using an electron microscope.

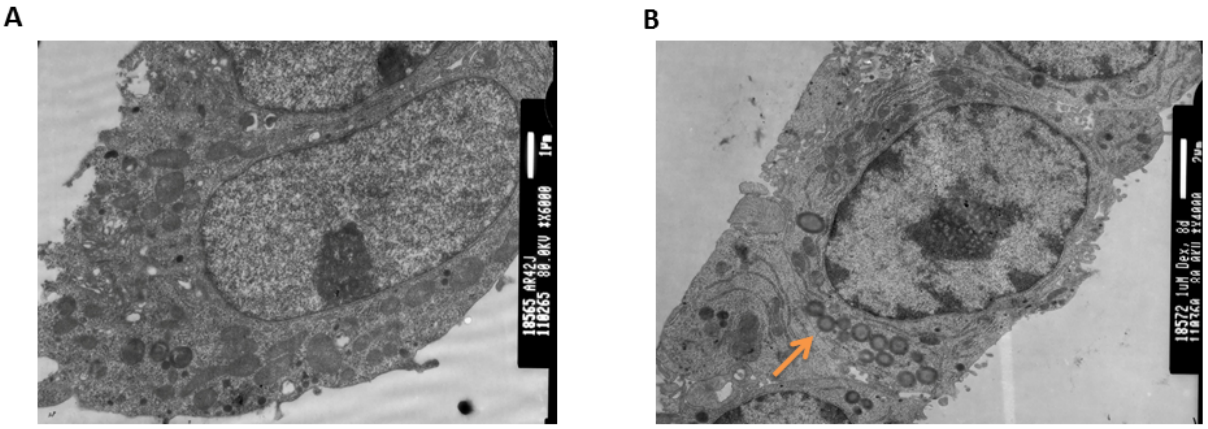

Fig.21 | The effect of dexamethasone on AR42J cells phenotype.

On the left side there is a cell before dexamethasone treatment (A), where we can observe lack of zymogen granules, and on the right side there are cells after 7days of 1μM dexamethasone pretreatment (B) with zymogen granules visible, marked with orange arrow.

The pretreatment of AR42J cells with 1μM dexamethasone result in formation of zymogen granules (Fig.21) and the rat acinar cells gains exocrine properties, so they can be used as a model in research about acute pancreatitis.

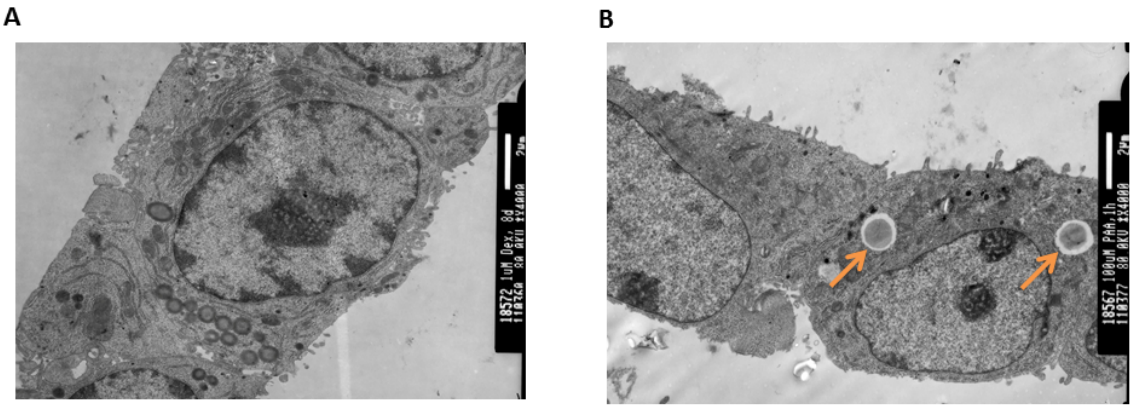

Fig.21 | The effect of 100μM peracetic acid treatment on AR42J cells phenotype.
On the left side we observe non-treated cell, established as control (A), and on the right side we observe cell after 1h incubation with 100μM peracetic acid (B). The orange arrows demonstrate the vacuoles.

In AR42J cells incubated for 1 h with 100μM peracid acid (PA), some vacuoles occurring have been observed containing homogeneous electron dense circular structure resembling lipid droplets. I observed no change in other cellular organelles.

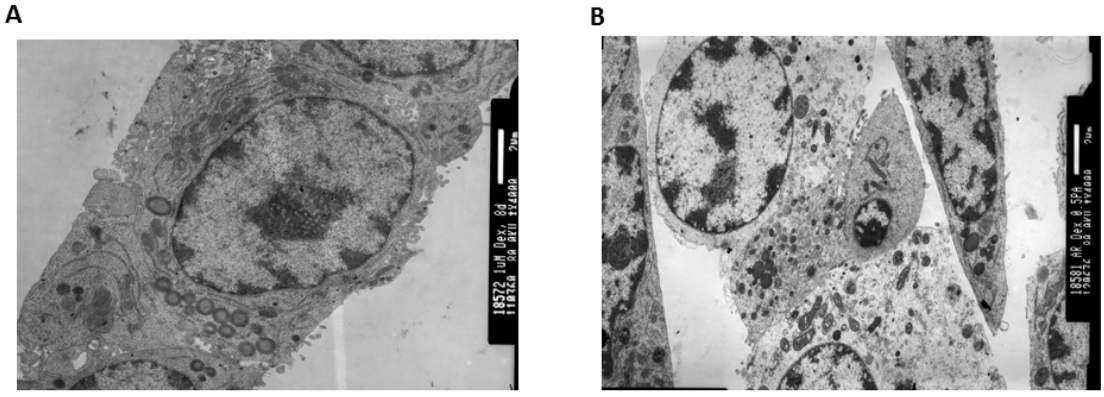

Fig.22 | The effect of 500μM peracetic acid treatment on AR42J cells phenotype.
On the left side we observe non-treated cell, established as control (A), and on the right side we observe cell after 1h incubation with 500μM peracetic acid (B).

AR42J cells treatment with 500μM peracetic acid for 1-hour result in cell swollen. Next to the intact cells, there are some others with evident changes – swollen nucleus, bright cytoplasm. Besides, we can also observe some cell fragments derived from cells that have collapsed.

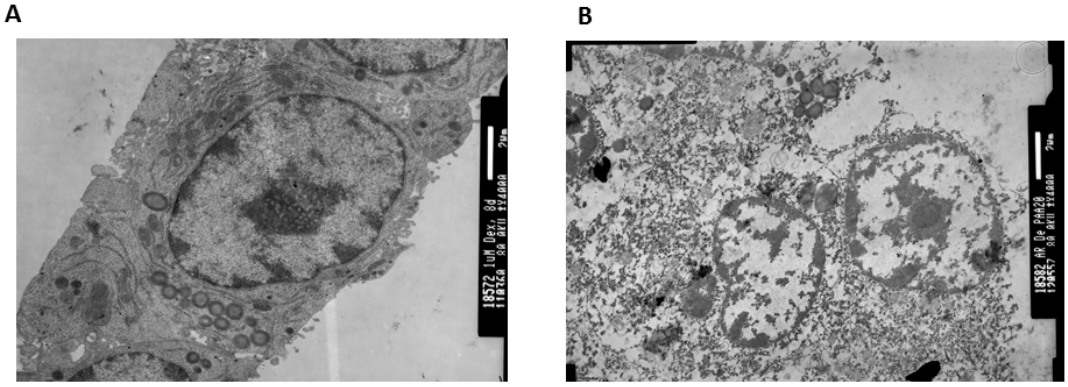

Fig.23 | The effect of 20mM peracetic acid treatment on AR42J cells phenotype.

On the left side we observe non-treated cell, established as control (A), and on the right side we observe cell after 1h incubation with 20mM peracetic acid (B).

The effect of 20mM PA treatment of acinar cells, result in total cell degradation. There is only nucleus outline visible, with condensed chromatin. The cell body collapsed and form semi-circular parts attached to cell membrane.

5. DISSCUSION

Oxidative stress is well established to increase throughout the course of pancreatitis. Furthermore, a hallmark of the inflammatory response in pancreatitis is the induction of cytokine expression, which is regulated by a number of signaling molecules including oxidant-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), signal transducer and activator of transcription 3 (STAT3), and mitogen-activated protein kinases (MAPKs). Cross talk between ROS and pro-inflammatory cytokines is mediated by NF-κB, AP-1, STAT3, and MAPKs; this crosstalk amplifies the inflammatory cascade in acute pancreatitis. Therefore, reducing the levels of ROS by antioxidant therapy may be clinically valuable for the treatment and prevention of acute pancreatitis. Depletion of pancreatic glutathione (GSH) has been shown to be involved in the early phase of acute pancreatitis [161] and also to influence the extent of disease severity [162]. The activities of multiple antioxidant enzymes, including glutathione peroxidase, superoxide dismutase (SOD), and catalase, decrease in the course of pancreatitis; the levels of antioxidant vitamins have also been shown to decrease [163,164]. It has been shown that peracetic acid has the ability to selectively oxidize ε-lysine residues. In SIRT3 there is cysteine Cys^{280}, which is crucial for this enzyme activity. In this study I want to suggest that inhibition of SIRT3 by peracetic (potential final metabolite of ethanol) leads to hyperacetylation of mitochondrial proteins, like Hsp60 or MnSOD. Inhibition of MnSOD by acetylation impairs ability of this protein to bind to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. This leads to increase ROS level, and oxidative stress, which in turn activate the result in premature digestive enzymes activation. Their activity in pancreas ended with whole organ damage, and necrotic changes of the tissue.

5.1. AR42J cell viability reduction after treatment with peracetic acid

It has been shown, that while ethyl alcohol metabolism, the hydrogen peroxide is produced. Hydrogen peroxide in the presence of carboxylic acids can lead to formation of peroxy acids – stronger oxidants. That is why in this study I suggested, that physiological occurring acetic acid combined with hydrogen peroxide lead to formation of peracetic acid, which is responsible for oxidative stress increase, and in turn starts the pancreatitis pathogenesis pathway.

In this study it has been shown that beyond acetic acid, all other treatment result in cell viability inhibition in concentration dependent manner. Peracetic acid, hydrogen peroxide and mixture of acetic acid with hydrogen peroxide (leads to formation of peracetic acid) shows statistically significant inhibition of cell growth (about 40%) after treatment with 250μM and more concentrated. That is why those concentrations were used in further investigations.

As it was shown in Fig.15, peracetic acid and mixture of hydrogen peroxide and acetic acid seems to be the most effective in cell viability inhibition of all tested compounds. PA and H_2O_2+AA in concentration 500μM show the inhibitory effect of more than 50%. The results of this study show that AR42J cells treatment with 500μM acetic acid which is consider to be the physiological metabolite of ethanol has no significant impact on cell viability, while treatment with 500μM PA or H_2O_2+AA induces about 60% loss of viability.

5.2. Induction of apoptosis using potential ethanol metabolites

Mechanisms of cell death in pancreatitis remain unknown. Parenchymal necrosis is a major complication of pancreatitis; also, the severity of experimental pancreatitis correlates directly with necrosis and inversely with apoptosis. Thus, shifting death responses from necrosis to apoptosis may have a therapeutic value. To determine cell death pathways in AR42J cells flow cytometric-double staining (Annexin V and PI) was performed. The results were surprisingly similar for both examined concentrations: 50μM and 500μM. In both cases all of the compounds shows induction of apoptosis. All the examined compounds in both concentrations, significantly increase apoptosis/necrosis level in comparison to control, untreated cells.

5.3. Level of ROS generation in AR42J cells raised after treatment with peracetic acid

The research on the pathogenesis of acute pancreatitis is the importance of oxidative stress caused by free oxygen radicals that arise as a result of the transformation of ethanol. Free oxygen radicals cause excessive activity of a leukocyte, which at the location of the organ disorders exacerbated oxido-reduction and causes the release of a number of proteolytic enzymes and thrombotic factors. As a result of the activation of inflammatory cells in the pancreas damage to the follicular cells, the release of lysosomal enzymes and organ destruction.

Present work demonstrated that the peracetic acid causes an increased production of ROS in relation to acetic acid and hydrogen peroxide alone. Already 50μM concentrations resulted in a statistically significant increase in ROS; however, in the case of ten times higher concentration change is meaningful and more evident by comparing the ROS for acetic acid and peracetic acid.

5.4. Treatment with peracetic acid leads to mitochondrial proteins acetylation of AR42J cells

Studies using immunoprecipitation methods have become a breakthrough during my research. The adopted hypothesis, took on a different meaning when it turned out that the treatment of AR42J cells with a peracetic acid significantly increases the level of acetylated forms of mitochondrial proteins, including Hsp60 and MnSOD.

During the treatment of the cells with 50μM peracid, I noticed a significant change in the level of acetylation of proteins in a sample using both peracetic acid and a mixture of acetic acid and hydrogen peroxide. In the case of protein Hsp60 change it is evident and clear and the results achieved, confirm my conviction of the rightness of the chosen ways of thinking about the pathogenesis of acute pancreatitis and provoke further investigations.

5.5. SIRT3 activity is inhibited by peracetic acid

Previously obtained results, confirming hyperacetylation of mitochondrial proteins in response to treatment with peracetic acid. Subsequent studies were made to verify the hypothesis, allegedly hyperacetylation Hsp60 and MnSOD is the result of inhibition of mitochondrial deacetylase SIRT3.

The kit was used to determine the inhibitory effect of all compounds tested. It has been shown that the concentration of 500μM of peracetic acid and mixture of acetic acid and hydrogen peroxide leads to a significant inhibition of SIRT3 of more than 100%. The results once again became a confirmation for the previously accepted hypothesis.

5.6. AR42J cell damage after treatment with ethanol metabolites

Using electron microscopy, it was possible to observe phenotypic changes within the pancreatic acinar cells, while treatment using peracetic acid.

In AR42J cells incubated for 1 h with 100μM peracid acid (PA), some vacuoles occurring have been observed containing homogeneous electron dense circular structure resembling lipid droplets. I observed no change in other cellular organelles.

AR42J cells treatment with 500μM peracetic acid for 1-hour result in cell swollen. Next to the intact cells, there are some others with evident changes – swollen nucleus, bright cytoplasm. Besides, we can also observe some cell fragments derived from cells that have collapsed.

The effect of 20mM PA treatment of acinar cells, result in total cell degradation. There is only nucleus outline visible, with condensed chromatin. The cell body collapsed and form semi-circular parts attached to cell membrane.

5. CONCLUSIONS

- Peracetic acid as well as combination of equimolar hydrogen peroxide and acetic acid lead to inhibition of acinar cells viability;

- Type of cell death seems to have no effect on molecular mechanism of action of peracetic acid in pathogenesis of acute pancreatitis;

- Peracetic acid significantly increase the level of ROS formation in acinar cells of pancreas, the same effect was observed using combination of equimolar hydrogen peroxide and acetic acid;

- Mitochondrial proteins undergo hyperacetylation after treatment with peracetic acid;

- Activity of SIRT3 is limited by peracetic acid;

- The AR42J cells undergo degradation while treatment with peracetic acid.

6. ACKNOWLEDGEMENTS

I am especially thankful to my supervisors, Professors Marianna Lauricella, Zbigniew Śledziński, Michał Woźniak and Francesco Cappello for their advice, patience and support throughout my PhD. It was an honor to be able to work with you. I am very grateful for the given opportunity to improve my research skills under your supervision.

I would like to thank all Medical Chemistry, Histology and Bioenergetics and Physiology of Exercise Departments Members from Medical University of Gdańsk - to all my colleagues for their ongoing help, technical support and work. Especially Dr Magdalena Górska for invaluable support in my research.

Words of appreciation also go to Dr Agata Zauszkiewicz-Pawlak for help with obtaining TEM photography.

Finally, I would like to thank my Family and Friends – for standing always by my side, I will never express with words how lucky I am to have all of you. Special thanks goes to my beloved husband Jarosław for his unceasing faith in me.

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