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Новые механизмы повреждения биологической ткани при низкотемпертурном воздействии *in vivo*

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New Mechanisms of Biological Tissue Injury Following the Low Temperature Exposure *In Vivo*

A number of theoretical and experimental studies, both *in vitro* and *in vivo*, have been performed to explain the action of low temperatures on tissue. It is now evident that the thermal parameters used in the past for freezing during cryosurgery were not precise; this may have resulted in the failure of treatment. For the first time, this report describes the early ultrastructural features of pancreatic parenchyma after low temperature exposure, i.e. cryosurgery, *in vivo*.

48 animals were used for the experiment. A temperature range of –80°C and –180°C in contact with pancreas tissue was selected for cryosurgical exposure. The cryosurgical response of pancreas parenchyma, i.e. ultrastructural cellular changes in pancreas tissue, was investigated. For transmission electron microscopy, the specimens from the pancreas parenchyma were taken immediately, one hour and 24 hours after the finishing of the freeze-thaw cycles intraoperatively.

The electronic microscopic analysis showed that, after local cryodestruction at temperatures of –80 and –180°C, similar processes occurred within the pancreas tissue in the early postcryosurgical phase – immediately and up to 24 hours after low temperature exposure on tissue. The exocrine pancreatic cells in the center of the cryozone changed upon thawing. Ultrastructural changes in the exocrine pancreatic cells, where the first signs of dystrophic processes had been noticed, were increased. These ultrastructural changes in the pancreatic cells provide a platform to better understand the mechanisms of damage and the pathogenesis of frostbite after cryosurgery. The properties of the pancreas parenchyma response after low temperature exposure provide important insights into the mechanisms of damage and the cryogenic lesion immediately after thawing in cryosurgery. Our new insights prove on the cell level that suddenly and progressively damaged pancreatic cells in the postcryosurgical zone lead to aseptic cryonecrosis and then to aseptic cryoapoptosis of vital normal tissue. The vascular capillary changes and circulatory stagnation demonstrate the antiangiogenesis mechanism, which, together with cryoaponecrosis and cryoapoptosis, are some of the main mechanisms of biological tissue injury following the low temperature exposure.

Key-words: cryosurgery, oncology, pancreas cell, antiangiogenesis, cryoaponecrosis, cryoapoptosis.

Важнейшая проблема при криотерапии – определение температурных параметров замораживания тканей. Оптимальные температуры в значительной степени исключают возможные неудачи при лечении. Практический интерес представляют исследования ранних свойств ультраструктуры паренхимы поджелудочной железы после воздействия низких температур, т.е. криохирургии *in vivo*.

В эксперименте использовали 48 животных. Для криохирургического воздействия при контакте с тканью поджелудочной железы был выбран температурный диапазон от –80 до –180°С. Изучали ответную реакцию паренхимы поджелудочной железы на криохирургию, т. е. изменения ультраструктуры клеток ткани поджелудочной железы. Для трансмиссионной электронной микроскопии образцы паренхимы поджелудочной железы брали сразу, через 1 час и 24 часа после окончания цикла замораживания-отогрева внутриоперативно.

Анализ с помощью электронной микроскопии показал, что после локальной криодеструкции при температурах –80 и –180°С сходные процессы происходили в ткани поджелудочной железы в раннюю посткриохирургическую фазу – сразу после низкотемпературного воздействия на ткань и вплоть до 24 часов. Экзокринные клетки поджелудочной железы в центре криозоны менялись после оттаивания; их ультраструктурные изменения обеспечивают платформу для лучшего понимания механизмов повреждения и патогенеза обморожения после криохирургии. Ответная реакция паренхимы поджелудочной железы после низкотемпературного воздействия представляет важную информацию в криохирургии о механизмах повреждения и криогенного патологического изменения сразу после оттаивания. Резкое и прогрессивное повреждение клеток поджелудочной железы в посткриохирургической зоне приводит к асептическому крионекрозу и затем асептическому криоапоптозу живой нормальной ткани. Сосудистые изменения капилляров и застой крови демонстрируют антиангиогенезный механизм, который вместе с крионекрозом и криоапоптозом являются основными механизмами повреждения биологической ткани после воздействия низких температур.

Ключевые слова: криохирургия, онкология, клетки поджелудочной железы, антиангиогенез, криоапонекроз, криоапоптоз.

Найважливіша проблема при кріотерапії – визначення температурних параметрів заморожування тканин. Оптимальні температури в значній мірі виключають можливі невдачі при лікуванні. Практичний інтерес представляють дослідження ранніх властивостей ультраструктури паренхіми підшлункової залози після впливу низьких температур, тобто кріохірургії *in vivo*.

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В експерименті використовували 48 тварин. Для кріохірургічного впливу при контакті з тканиною підшлункової залози був обраний температурний діапазон від –80 до –180°С. Вивчали відповідну реакцію паренхіми підшлункової залози на кріохірургію, тобто зміни ультраструктури клітин тканини підшлункової залози. Для трансмісійної електронної мікроскопії зразки паренхіми підшлункової залози брали відразу, через 1 годину і 24 години після закінчення циклу заморожування-відігрівання внутрішньоопераційно.

Аналіз за допомогою електронної мікроскопії показав, що після локальної кріодеструкції при температурах –80 і –180°С подібні процеси відбувалися в тканині підшлункової залози в ранню посткріохірургічну фазу – відразу після низькотемпературного впливу на тканину аж до 24 годин. Екзокринні клітини підшлункової залози в центрі кріозони змінювалися після відтавання, їхні ультраструктурні зміни забезпечують платформу для кращого розуміння механізмів ушкодження і патогенезу обмороження після кріохірургії. Відповідна реакція паренхіми підшлункової залози після низькотемпературного впливу представляє важливу інформацію в кріохірургії про механізми ушкодження і кріогенної патологічної зміни відразу після відтавання. Різке і прогресивне ушкодження клітин підшлункової залози в посткріохірургічній зоні приведе до асептичного кріонекрозу і потім асептичному кріоапоптозу живої нормальної тканини. Судинні зміни капілярів і застій крові демонструють антиангіогенезний механізм, що разом із кріонекрозом і кріоапоптозом є основними механізмами ушкодження біологічної тканини після впливу низьких температур.

Ключові слова: кріохірургія, онкологія, клітини підшлункової залози, антиангіогенез, кріоапонекроз, кріоапоптоз.

The use of deep freezing as a method of destroying biological tissue has been known for a long time and has been described in detail [1–9]. Cryosurgery is based on the application of low temperatures to destroy abnormal tissue. The use of cryogenic techniques for the resection of parenchymal organs [10–12] and *in situ* ablation of tumors using subzero temperatures for surface malignancies [13–15] have been described. Advances in this technology have aroused interest in the use of cryosurgery for the treatment of parenchymal malignancies [16–17].

Tumors are not cut out but shock frozen. Recent scientific research on the application of freezing in the fields of biology and medicine, and numerous theoretical and experimental studies *in vitro* and *in vivo*, have demonstrated the effect of low temperatures on tissue as well as the impairment and destruction of cells under cryoinfluence [18–22].

Advances in cryosurgical technology have further stimulated interest in using low temperatures to treat diseases of the pancreas, first and foremost pancreas malignancies [23–28]. It has been suggested that cryoablation should be used where pancreas tumors are deemed unresectable [29–30].

The pathophysiologic consequences of freezing a significant portion of primate pancreas have been investigated using a liquid nitrogen cryosurgical probe [31]. Histologically, typical cryonecrosis occurred in the treated area, while untreated pancreas and surrounding tissue remained normal. This study demonstrates that cryonecrosis of a significant portion of pancreatic tissue can be produced in a primate without the development of diffuse pancreatitis or other ill effects.

The other study was designed to evaluate the effects of direct pancreatic surface cooling on the exocrine pancreas [32]. The changes in serum amylase levels, pancreatic water, amylase and cathepsin B as a lysosomal enzyme, content, histological changes of acinar cells, and the subcellular distribution of cathepsin B after 1–2- and 3-hours of direct pancreatic cooling in rats have been measured. In additional, the in-vivo amylase and cathepsin B output stimulated by caerulein, *in vitro* lysosomal and mitochondrial fragility as well the pancreatic adenylate energy metabolism were evaluated.

Electron microscopy of acinar cell of the pancreas cooled to –30°C with subsequent thawing has already been reported [33].

Quick-freezing and freeze-drying were used in preparation for high quality morphology and immunocytochemistry at the ultrastructural level to obtain ultrastructural localization of insulin in the pancreatic beta cell by the unlabeled antibody-enzyme technique [34].

Also, fetal rat pancreases, cultured for 8 days in PRMI 1640, were successively frozen to –196 degrees C [35]. The effects of culture, various cooling times, warming rates, thawing procedures, dimethyl sulfoxide concentration, and ultrastructural changers of cellular elements were analyzed.

Currently no data is available either on ultrastructural cellular changes of cryosurgical response in normal and pathological pancreas tissue when exposed to low temperatures.

Neither the *in vivo* sensitivities of the pancreas parenchyma to different cryosurgical exposures, nor the minimum temperature required to cause adequate cryodestruction and prevent tumor recurrence, have as yet been defined or described in medical literature. The effect of different temperature applications on the freeze-thawing process and the cryosurgical response of normal pancreas tissue in animals have not been investigated by electron microscopy. Such studies would permit an assessment of cryosurgical techniques as a treatment option for diseases of the pancreas, especially for pancreatic tumor.

To our knowledge, this is the first study investigating the ultrastructural findings in the acinar cells of the animal pancreatic parenchyma after exposure to different low temperatures. An experimental study in pancreas cryosurgery, revealing hitherto unreported ultrastructural changes in the exocrine cells of the dog's pancreas after the freeze-thawing process, is presented here.

Materials and Methods

Animals. The experiment was carried out on 48 mongrel dogs weighing 12.1 to 14.6 kg. The aim of the study was to investigate the processes which occur in pancreas parenchyma subjected to destruction by the application of low temperatures of various intensities and the degree of their early ultrastructural manifestations.

Experimental cryogenic destruction was performed as follows: After anesthesia, an upper medial laparotomy was performed and the abdominal cavity was opened in layers. The pancreas was brought as close to the opening as possible. The anterior surface of the organ was exposed and the cryogenic instrument of the Cryoelectronic (Scientific-Production Company "Pulse", Kyiv, Ukraine) device was firmly applied to the organ [36].

The operation control unit was adjusted to the temperature required to produce the cryogenic effect of –80°C and of –180°C because the cryozone formation is dependent on different parameters, particularly on these temperatures. This way an *in vitro* experimental study was shown [37]. Cryogenic destruction which means only freezing was performed for three minutes. The time parameter was investigated in this study too [37]. The freeze-thaw cycle was monitored using intraoperative ultrasound before, during and after cryosurgery. The cryogenic applicator was then automatically warmed up and removed from the tissue.

Automatic thawing of the destruction area was continued up to four minutes. In order to study early ultrastructural changes in the pancreas parenchyma after low temperature exposure, biopsy samples were taken immediately, one hour and 24 hours after warming up. For transmission electron microscopy, the specimens were taken immediately and one hour after the finishing of the freeze-thaw cycles intraoperatively. Afterwards, the abdominal cavity was closed. Further, the next specimens were taken in 24 hours, this time also intraoperatively.

A total of 48 healthy adult mongrel dogs of both sexes weighing 12.1 to 14.6 kg were used for the experiment. All animals were quarantined at the University Animal Care Facility for one week, and the dogs were kept strictly in accordance with the guidelines for the care and use of research animals established by the animal ethics committee of the National Medical University and the Ukrainian National Veterinary University in Kyiv. All animals were provided with standard pellet food and water, but were not fed for 12 hours prior to the experiment. In this study, all dogs were kept in their cages and observed for next 24 hours postoperatively.

Experimental Design. Twenty-eight animals were used for the experiment. The dogs were divided into two groups of fourteen each. In group A the pancreatic parenchyma was frozen to –80°C and in group B to –180°C. The cryoprobes were in contact with the animal pancreas for three minutes at each of these temperatures. The remaining twenty animals were used as controls and again divided into two groups. In the first control group (10 animals) the cryoprobe was inserted into the pancreas but no freeze-thaw cycle was conducted. The second control group of ten animals underwent sham (simulated) laparotomy (opening of the abdominal cavity).

Operative Procedure. After a twelve-hour fast the animals were anesthetized by intravenous injection of 50 mg/ml ketalar (0.2 mg/kg body weight) and xyla (xylazine base, 0.2 mg/kg body weight) in a ratio of 1:1 (InterChemie, Netherlands). After endotracheal intubation the animals were ventilated with a respirator. The pancreas was exposed for laparotomy using an oblique abdominal section and a vertical or hockey-stick-shaped incision towards the left costal arch. The pancreas was examined bimanually and the peritoneal cavity was explored to rule out extrapancreatic disease. The pancreas was then isolated by disconnecting all of its peritoneal attachments. One hour after cryo-surgery the abdominal wall was closed in two layers. In 24 hours, this operative procedure was repeated. When the dogs regained consciousness they were returned to their cages.

The Cryosurgical Approach. A disc probe with a diameter of 20 mm was placed on the pancreas. A temperature range of (–80°C) and (–180°C) in contact with pancreas parenchyma, at a temperature stabilization of ±1%, was selected for cryoexposure. A three-minute freeze followed by complete thawing of the pancreas was used for each freeze-thaw cycle. Every cryolesion was observed for 60 minutes after thawing. The two freeze-thaw cycles were monitored by intraoperative ultrasound before, during and after cryosurgery. Liquid nitrogen was applied by Cryoelectronic, a cryosurgical device of our own, produced by the Scientific Company (Kyiv, Ukraine), suitable for universal application. Liquid nitrogen was circulated through the probe at –196°C [36].

Freezing with a Cryoprobe. Specific lesions in the pancreas could be identified, targeted, and cryoablated in the animal experiment. A cryoinstrument with a disk-shaped cryoprobe measuring about 20 mm in diameter was placed on the pancreas tissue to induce necrosis (tissue destruction). Guided by continuous intraoperative ultrasonic monitoring, the pancreas tissue was frozen to –80°C and –180°C in order to achieve complete tissue destruction. The ice-ball (cryogenic zone) was generated without difficulty. The line of demarcation between the area that was cryosurgically destroyed and followed by cryogenic necrosis, and the healthy pancreatic parenchyma, is clearly outlined by the rim of the cryozone. The cryozone includes a 7 mm margin which surrounds the normal-looking

pancreas parenchyma. When the pancreas tissue had frozen to an ice-ball at a temperature of –80° and –180°C, liquid nitrogen refrigeration was stopped.

Immediately after a single freeze-thaw cycle using a cryoprobe with a diameter of 20 mm, the cryozone measured 34 mm. During the freezing process the diameter of the cryogenic zone was 7 to 15 mm larger than that of the cryoprobe. No intraoperative or postoperative complications were encountered. All dogs tolerated pancreas cryosurgery well and none died.

Thawing the Tissue. Thawing each freeze-thaw cycle took approximately 4 minutes in the automatic cryosurgical unit. The cryoprobe was warmed up and removed from the tissue. The two freeze-thaw cycles that were performed for each cryolesion in the course of expe-rimental cryosurgery revealed the same cryozone with an ice crater in the middle and an ice margin with a demarcation line immediately after cryosurgery.

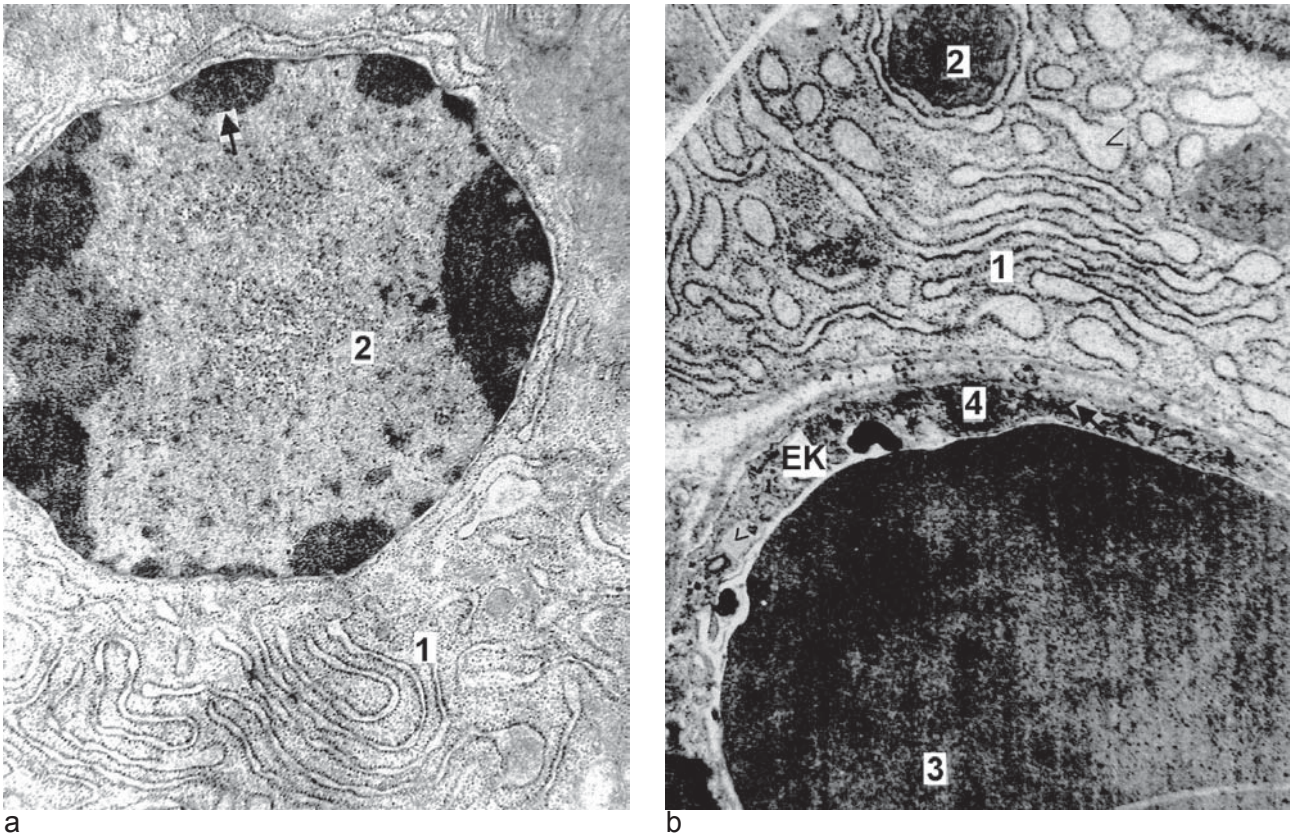
Electron Microscopic Study of the Pancreas. For transmission electron microscopy, the specimens of the pancreas tissue were divided into fragments and then secured in a 1% solution of osmium tetraoxide for a period of two hours at a temperature below 4°C. The objects were dehydrated for fifteen minutes in each of the following solutions: a) 70° and 80° ethanol at 4°C, b) a room temperature (18°C) of 96° ethanol, c) 3 portions of 100° ethanol, d) in a mixture of ethanol and acetone, e) in 2 portions of acetone. The objects were immersed in 3:1, 1:1, 1:3 mixtures of acetone and epoxy resin.

They were left in each of the mixtures for one hour, in pure resin for 12 – 24 hours and then embedded in epoxy resin. The latter consisted of an Epon-Araldite mixture. Section of 0.5-microm thickness were cut and then stained with 2% solution of uranyl acetate on 70° ethanol for 15 minutes, and left in a lead citrate(s) concentration for a further 15 minutes.

For light microscopic studies, specimens from the pancreas parenchyma were collected from the cryozone immediately, one hour and 24 hours after cryosurgery. The biopsy was taken from the margin between frozen and normal pancreas parenchyma, i.e., from the center of the cryonecrosis, and investigated under a light microscope. The histological results have been published separately.

Results

The Freeze-Thaw Cycle at –180°C. For the first time, tissue was taken directly from the central portion of the cryozone in the dog's pancreas immediately after thawing to observe the ultrastructural changes in pancreas parenchyma (Fig. 1a). An exocrine pancrea-



a b

Fig. 1. a – freeze-thaw cycle at –180°C: Exocrine pancreatic cell (1), nucleus (2), margination of the chromatin into large aggregates (↑), canaliculi of the rough endoplasmic reticulum (∠); **b –** pancreatic exocrine cell (1), mitochondria and a small number of cristae (2), canaliculi (∠), erythrocytes (3), endothelium cell (4), canaliculi of the endoplasmic reticulum (∧), basement membrane (↑).

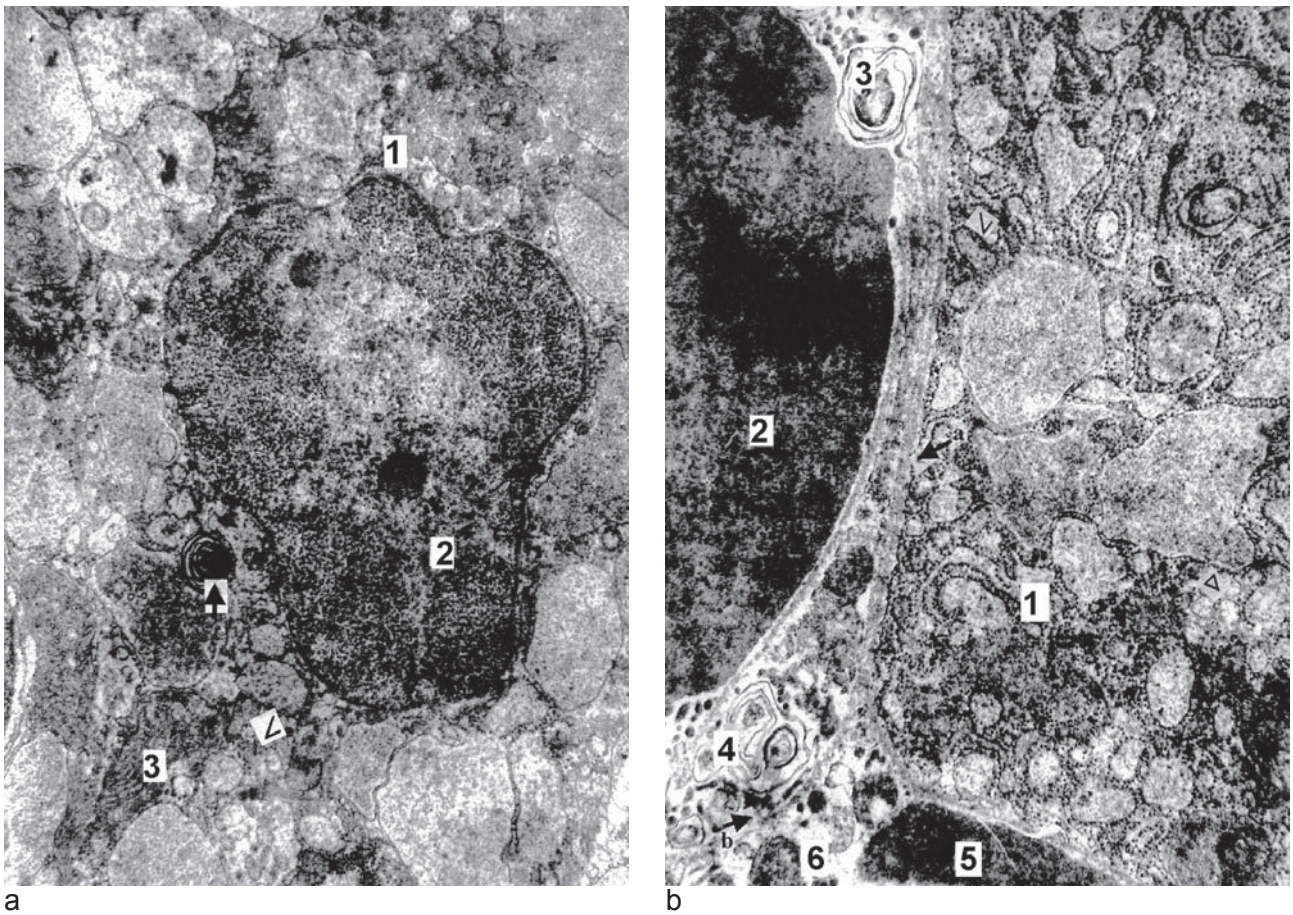


Fig. 2. a – pancreatic exocrine cell (1), nucleus (2), mitochondria (3), dilated canaliculi of endoplasmic reticulum (∠), myelin-like structures (↑); **b** – pancreatic exocrine cell (1), plasmatic membrane (↑a), canaliculi (∠), erythrocyte (2), myelin-like structure (3), endothelial structure (↑b), myelin-like structure (4), cell organelle (6).

tic cell (1) and the nucleus (2) with a well preserved nuclear envelope (membrane) are seen. The perinuclear space is insignificantly dilated all over the cell. The chromatin is divided into large aggregates (↑). The canaliculi of the rough endoplasmic reticulum are slightly dilated and partly fragmented (∠). Zymogen granules are absent.

As demonstrated by Fig. 1b, the bottom part of the pancreatic exocrine cell (1), mitochondria with a dense electronic matrix, and a small number of cristae (2) are seen. Canaliculi of the rough endoplasmic reticulum are slightly dilated and partly fragmented (∠).

Erythrocytes (3) are found in the vascular capillary space. An endothelium cell (EK) with electron-dense mitochondria (4), canaliculi of the endoplasmic reticulum (∧) and ribosomes are visible. The basement membrane (↑) of the endothelium cell is reduced to powder.

Fig. 2a shows the central portion of the cryozone in the dog's pancreas one hour after thawing. An exocrine pancreatic cell (1) with a nucleus (2) possessing a dense chromatin and mitochondria with an electron-dense matrix, and a small number of cristae, are seen. Dilated canaliculi of the endoplasmic reticulum full of an amorphous flake-like substance (∠) are also observed. No ribosomes are found on the membranes. The number of ribosomes in the cytoplasm is suddenly reduced. The myelin-like structures (↑) in the cytoplasm testify to the increase in free radicals.

Also, Fig. 2b illustrates the central portion of the cryozone in the dog's pancreas one hour after thawing. An exocrine pancreatic cell (1). The plasmatic membrane on the bottom (↑a) surface is not clearly structured. Canaliculi of the endoplasmic reticulum (∠) fragmentize upon intensification of the edema and loosen the ribosomes. An erythrocyte (2) and a myelinlike structure (3) are present in the vascular capillary space where the endothelial structure is locally damaged (↑b). The endothelial cell reveals a myelin-like structure (4). An erythrocyte (5) and the remains of the cell organelles (6) in the interstitial space are also seen.

Fig. 3a illuminates the central portion of the cryozone in the dog's pancreas 24 hours after thawing. An exocrine pancreatic cell (1) with a pyknotic nucleus is seen. The cytoplasm is filled with an electron-dense homogeneous content (3) and with the canaliculi of the endoplasmic reticulum (↑). A segmented neutrophil is seen in the center of the inflammation (4).

Twenty-four hours after thawing. The central portion of the cryozone in the dog's pancreas after

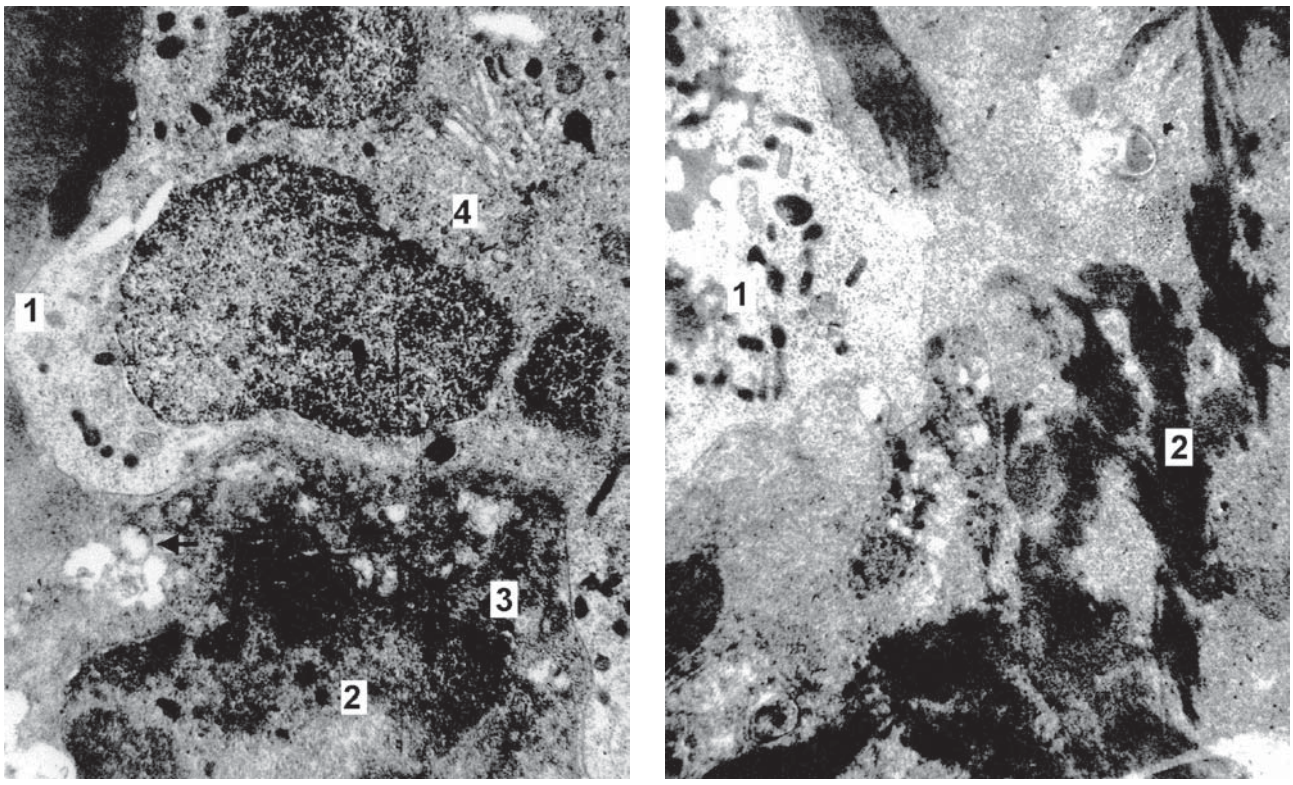


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Fig. 3. a – pancreatic cell (1), pyknotic nucleus (2), cytoplasm (3), endoplasmic reticulum (↑), neutrophil (4); **b –** Cell detritus (1), fibrin fibers (2).

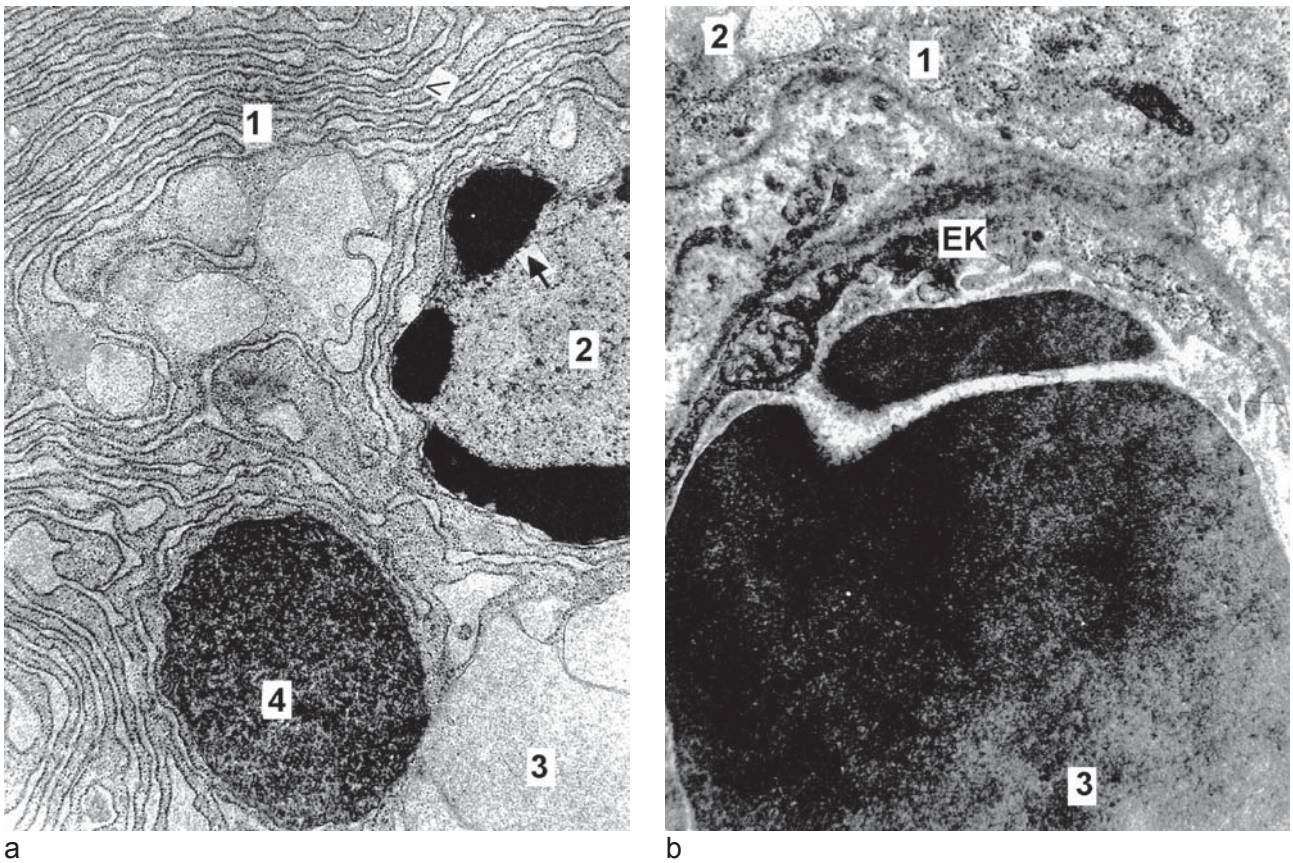

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Fig. 4. a – freeze-thaw cycle at –80°C: Pancreatic cell (1), nucleus (2) with aggregates (↑), endoplasmic reticulum (∠), ribosome (3), homogeneous substance (4); **b –** pancreatic cell (1), membrane (↑), endoplasmic reticulum (2), erythrocyte (3), endothelial cell (EK).

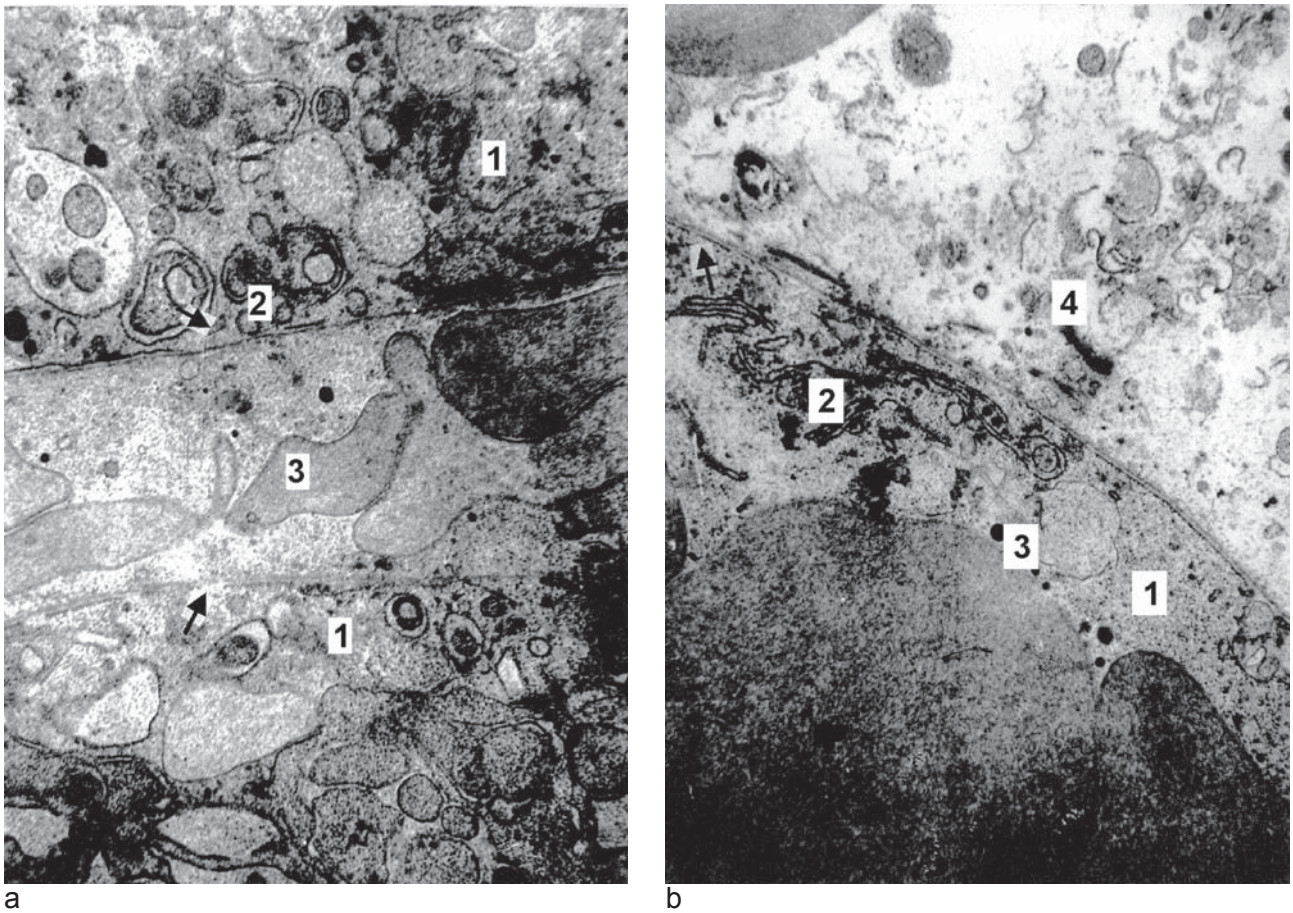

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Fig. 5. a – exocrine pancreatic cell (1), plasmatic membrane (↑), endoplasmic reticulum (2), erythrocyte (3); **b** – pancreatic cell (1), plasmatic membrane (↑), endoplasmic reticulum (2), homogeneous substance (3), cell detritus (4).

thawing (Fig. 3b), cell detritus (1) and fibrin fibers (2) are observed.

Freeze-Thaw Cycle at –80°C. The central portion of the cryozone in the dog's pancreas after thawing. Tissue was taken immediately afterwards (fig. 4a). The illustration reveals an exocrine pancreatic cell (1) and the nucleus (2) with the sharply outlined rim of the chromatin and the formation of aggregates (↑). Undilated and dilated canaliculi of the rough endoplasmic reticulum (∠) with loose ribosomes (3) are visible. Zymogen granules are absent. The electron-dense homogeneous substance (4) is reduced.

Fig. 4b demonstrates the central portion of the cryozone in the dog's pancreas; here the tissue was extracted immediately after thawing. An exocrine pancreatic cell (1) is well seen.

The plasmatic and basement membranes (↑) of the cells from the side of the basement surface have been preserved. There are countless ribosomes and insignificantly dilated canaliculi of the endoplasmic reticulum (2). Furthermore, erythrocytes (3) are present in the vascular capillary space, and the endothelial cells (EK) of the vascular capillary are largely preserved.

Fig. 5a illustrates the central portion of the cryozone in the dog's pancreas one hour after thawing. An exocrine pancreatic cell (1) is visible. The plasmatic membrane (↑) of the cells on the bottom surface is locally lysed. Canaliculi of the rough endoplasmic reticulum (2) are filled with an amorphous flake-like material of a different electronic density. Empty erythrocytes (3) are seen in the interstitial space.

Fig. 5b shows the central portion of the cryozone in the dog's pancreas 1 hour after thawing. An exocrine pancreatic cell (1) and a plasmatic membrane (↑) which are locally lysed, can be clearly observed. Canaliculi of the rough endoplasmic reticulum (2) are seen.

Drops of an electron-dense homogeneous substance are present (3). Cell detritus (4) is also seen in the interstitial space.

Fig. 6a elucidates the central portion of the cryozone in the dog's pancreas 24 hours after thawing. Cell detritus (1) has formed instead of the exocrine pancreatic cells. The damaged blood cells (2) in the vascular space together with the endothelium cover are fully desquamated and a crumbly basement membrane (↑) is seen.

Finally, (Fig. 6b) illustrates the central portion of the cryozone in the dog's pancreas 24 hours after thawing. Damaged exocrine pancreatic cells (1) are seen.

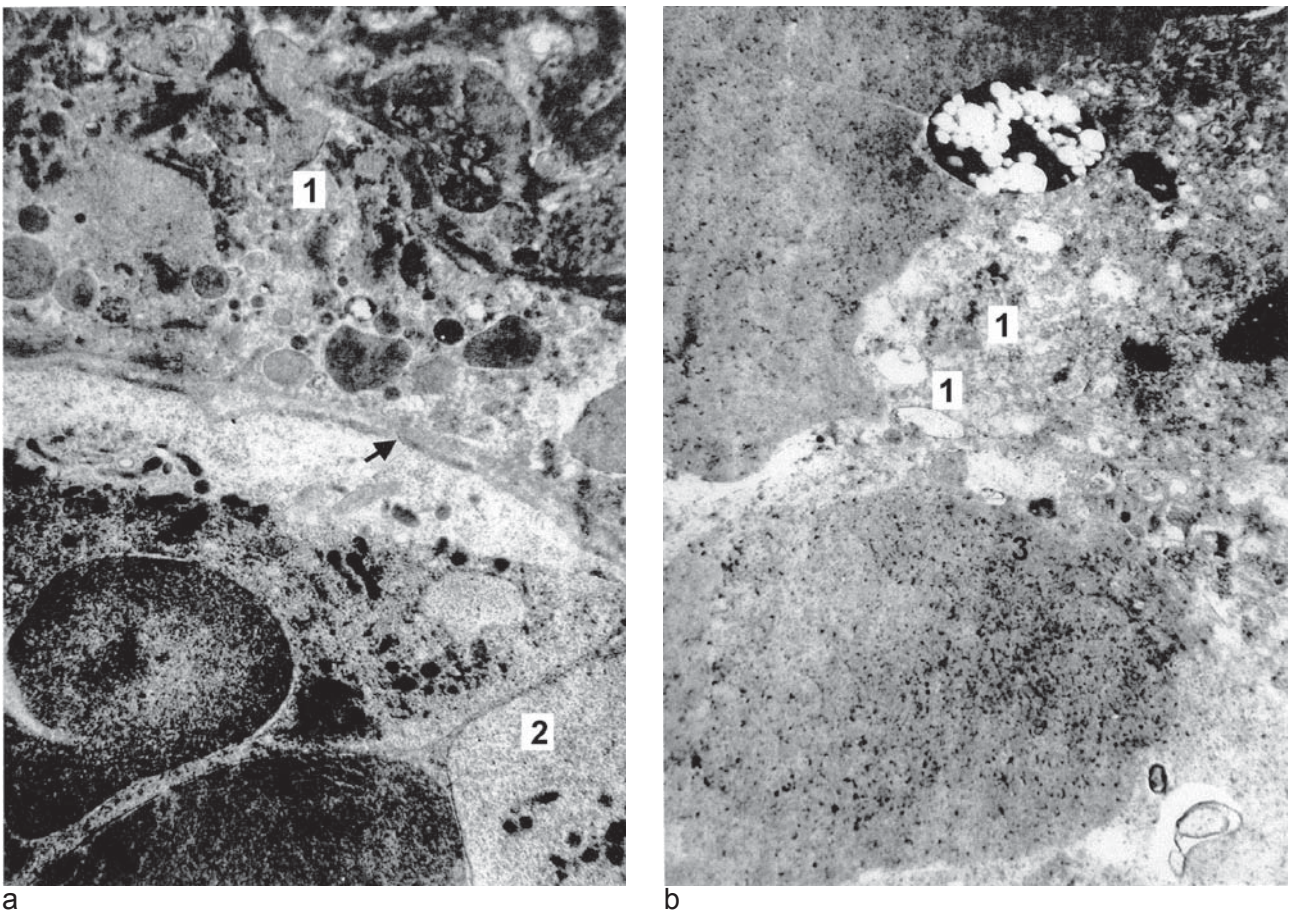

Fig. 6. a – cell detritus (1), blood cell (2), membrane (↑); **b –** 24 hours after thawing: exocrine cell (1).

Discussion

The study has been carried out on the effects of low temperature exposure of various intensities on pancreas cells. Our results clearly suggest that the pancreas cell damage after freezing and thawing of various intensities is of acute type and the pancreas cell architecture can not be return more or less to normal. The disruption of pancreas cells has been occurred after freezing and thawing using different low temperatures, especially at temperatures of –80°C and –180°C. Pancreas cells are entities with a highly specific intracellular morphological content, separated from the non-specific extracellular solution by the cell membrane.

An inadequately addressed aspect of cryosurgery is the mechanism by which damage is affected during the freezing process. This is important because, although cryosurgery is clinically used to treat parenchymal tumors, especially liver tumors, neither the freezing process nor the mechanism of damage involved in this kind of treatment are fully understood.

Therefore, research studies on the process of freezing in liver tissue are of great significance for experimental studies in pancreas surgery.

An experiment-based investigation of pancreas cryosurgery must address the following aspects: 1) the mechanism of tissue destruction with respect to pancreas freezing; 2) the viability and safety of cryosurgical applications in the pancreas; 3) biological perspectives of freezing pancreas tumors; and 4) experimental studies focused on refining the technology of the equipment used for pancreatic cryosurgery.

The data presented here constitute the results we obtained from the experimental investigations in the field of pancreas cryosurgery. In the present study, healthy pancreas parenchyma was investigated grossly and morphologically by electron microscopy. The study was focused on the mechanism of the freeze-thawing process with applications of -80°C and of -180°C to pancreatic tissue by means of early pancreatic cell changes. Endothermic transformation of the cryogenic lesion in the pancreas parenchyma already immediately, one hour and 24 hours after freeze-thawing does correlate with various low temperatures.

In this study, the following early ultrastructural changes in the formed postcryosurgical zone occur in the pancreas at the temperature of –180°C more than at the temperature of –80°C: the division of chromatin into large aggregates, the dilation and partly fragmentation of the canaliculi of the rough endoplasmic reticulum, the reduction of the basement membrane of the endothelium cell, the reduction of the number of ribosomes, and the presence of the myelinlike structures in the vascular capillary space. Further, the cell

detritus and the fibrin fibers are more clearly visible at the temperature of –180°C, compared to –80°C.

Cell changes in the pancreas tissue augment with time both in animals after exposure to –180°C and in animals after exposure to –80°C. Immediately after the freeze-thaw cycle at –180°C, no myelin-like structures in the cytoplasm can be found. They occur only after one hour after thawing, indicating an increase in free radicals. On the other hand, a segmented neutrophil as well as cell detritus and fibrin fibers are not observed immediately nor one hour after the freeze-thaw cycle, but only after 24 hours.

Similarly, in this study, the cell changes in the pancreas tissue depend on time after the freezethaw cycle at –80°C. Thus, a cell detritus in the interstitial space can firstly be seen one hour after thawing in the cryozone and damaged exocrine pancreatic cells are visible 24 hours after thawing in the dog's pancreas.

Further, our investigation demonstrated that the damaged endothelial structure and the damaged blood cells in the vascular capillary space including segmented neutrophils are the initial signs of an aseptic inflammation and circulatory stagnation after the freeze-thaw cycle.

The results of our study have shown that the early pancreatic cell changes in the postcryosurgical zone being observed in the first 24-hours after the freeze-thawing cycles are the beginning phase in the whole postcryosurgical process [38]. They lead to the next phase of aseptic cryoaponecrosis and then to aseptic cryoapoptosis which, in many weeks, will clinically finish by the formation of a postcryosurgical crust and a postcryosurgical scar.

Further, fundamental investigations of cryodestruction mechanisms in pancreatic tissue would allow the researcher to list the main requirements of cryosurgical equipment. The main technical parameter for effective cryodestruction is the provision of a sufficiently low, subzero temperature in the biological tissue, followed by deliberate thawing. Our findings, i.e. early ultrastructural changes in the pancreas parenchyma, show that research into cryosurgical techniques will yield data on the main medical requirements for the application of cryomethods (39). However, studies which will reflect the late ultrastructural changes in the pancreas after low temperature exposure, e.g. one week, two weeks, four weeks, two months, three months, six months, are yet to be conducted. Such data would enhance the efficiency of cryosurgical methods in the different fields of medicine, primarily for the treatment of malignant tumors in the pancreas and other parenchymal organs. Thus, our findings and additional further studies shall fully understand the ultrastructural features of parenchymal tissue after cryosurgery, giving rise to a new concept concerning the technical requirements of cryosurgical equipment to perform modern cryosurgical operations, especially in oncology.

The optimal technical data for the modern universal cryosurgical systems should be of a high cooling capacity, of a great accuracy, this way guaranteeing the cryo-destruction of the specified volume [40].

Prospective randomized clinical trials concerning cryogenic surgery and conventional surgical techniques in patients with pancreas carcinomas have not been published so far. It is, however, necessary to work out a clinical concept of cryosurgical and conventional surgery focused on intraoperative tumor reduction in pancreatic cancer, as this would improve the patients' survival rate and quality of life.

Conclusions

The present observations on the early ultrastructural changes in the pancreas tissue clearly provide a platform to better understand the mechanisms of damage and the pathogenesis of frostbite during cryosurgery. The properties of the pancreas parenchyma response after low temperature exposure provide important insights into the mechanisms of damage and the cryogenic lesion immediately after thawing in cryosurgery. Progressively damaged pancreatic cells in the postcryosurgical zone lead to cryoaponecrosis and cryoapoptosis. Vascular changes and circulatory stagnation indicate the anti-angiogenesis mechanism of biological tissue injury after low temperature exposure to –180°C and –80°C. Thus, cryoaponecrosis, cryoapoptosis and anti-angiogenesis are some of the most important mechanisms of living tissue damage as a response to cryosurgery.

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