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The histological and ultrastructural picture of the paraepidermoidal epithelium subjected to low temperatures. I

Obraz histologiczny i ultrastrukturalny nabłonka paraepidermoidalnego poddanego działaniu niskich temperatur. I

The action of low temperatures inside the tissues considerably depends on the vascularization and haemodynamics of the tissue. There is an increase in the temperature from the centre of the action of low temperature in the direction of circumference which comes from blood vessels in deeper layers. This fact, as well as other heat conductivity capacities specific for a given tissue, leads to the creation of ice crystals far from the centre of freezing.

The irreversible damage of cells is solely attributed to the range of temperatures from -5^0C to -50^0C. Further reduction of temperature does not intensify cryodestruction phenomenon, however, the rate of the freezing of tissues is most essential. In gradual freezing (e.g. 10^0C per minute) first extracellular water turns into ice and the endogenic water goes only through the phase of hypothermia.

In fast freezing (e.g. 100^0C per minute) the exchange of water and electrolytes between intra - and extracellular spaces is limited, the consequence of which is the regular creation of ice crystals in both spaces that directly leads to the cell destruction (4).

Presently used apparatus for freezing of tissues makes it possible to obtain low temperatures fast which causes the formation of little ice crystals. The size of ice crystals diminishes proportionally to the decrease in temperature, it reaches the molecular size (10 to 25 Å) at the temperature of -100^0C so that the ice crystals are able to get inside the cell through the cell membranes unhampered. Cryosurgical cell destruction caused by

the fast reduction of temperature reaches its peak during the following defreezing phase. During the spontaneous defreezing process which is three or four times longer than freezing, in the range of temperatures from –40°C to –3°C there appears the so-called recrystallization. The consequence of this physical effect is the transformation of small ice crystals into the bigger ones, which causes the additional mechanical damage of the cell membrane. The cycle freezing-defreezing-freezing leads to the worse cell damage than a single freezing (9). Long-term world research on the mechanism of impact of low temperatures on the living organism tissues has not brought any explicit explanation of this process so far. Because of the increase in indications for cryosurgical operations as a therapeutic procedure in different cases of diseases of the oral cavity and face, different techniques of cryoapplication were elaborated and introduced. They depended on the size of the probe, its temperature and duration of the freezing.

Additionally, there appeared the need of anticipation and thorough planning of the size of space subjected to cryodestruction and also the investigation stating on what depth of tissue first irreversible changes appear in cells due to freezing.

MATERIAL AND METHODS

The material for the research was the epithelium of the clinically unchanged oral vestibule mucosa collected from patients of the Clinic of Dental and Maxillofacial Surgery of Medical University of Lublin, following their previous consent during various surgical operations.

In the total number of 21 patients there were 10 men aged 28 to 76 and 11 women aged 17 to 67. The operations were carried out with the use of cryosurgical apparatus called AUK-20A, constructed in the Centre of Medical Technique in the Department of Low Temperatures in Warsaw. In this procedure, cylindrically shaped metal, closed applicator was used. It was 10mm in diameter and it was cooled with liquid nitrogen with the temperature of –196°C. The tissue was being frozen in the time of 1, 2, and 5 seconds.

Four segments of the epithelium of the oral vestibule mucosa were collected from each patient for the microscopic examination. The segments were 2 mm long and 1 mm wide and they were collected in the following order: 1. The segment of the epithelium of the clinically unchanged mucosa as a control segment; 2. The segment of the epithelium of the clinically unchanged mucosa frozen with the temperature of –196°C within 1 second, with the use of cryoprobe 10mm in diameter, collected directly after freezing; 3. The segment of the epithelium of the clinically unchanged mucosa frozen with the temperature of –96°C within 2 seconds, with the use of cryoprobe 10mm in diameter, collected directly after freezing; 4. The segment of the epithelium of the clinically unchanged mucosa frozen with the temperature of –196°C within 5 seconds, with the use of cryoprobe 10mm in diameter, collected directly after freezing.

All segments were preliminary fixed for 3 hours at the temperature of 4°C in 4% glutaraldehyde, then they were fixed again in 1% osmium tetroxide for 2 hours in the temperature of 4°C. The segments were dehydrated in ethanol, conducted through the propylene oxide and embedded in Spurr Low-Viscosity Kit of Polysciences Company USA. The material was cut with the use of Richert Om U3 ultramicrotome.

The semithin sections 1.5 to 2 microtome thick were stained with 1% azure II and 1% methylene blue in 1% sodium borate and observed in the light microscope. The semithin sections were stained once more with uranyl acetate and lead citrate according to Reynolds method. The ultrathin preparations were observed in the electron microscope type BS 500 Tesla.

RESULTS

Normal epithelium that was not exposed to the cryoapplication /group I/ reveals characteristic arrangement and shape of cells in its entire cross-section. The basal layer cells reveal oblong shape and their cell axis is more or less perpendicular to the basement membrane. Among basal layer cells only single cells were observed with brighter and more abundant cytoplasm which could correspond with melanocytes. Progressing to the surface of the epithelium, the cells assume polygonal shape and next they flatten. The cytoplasm of the prickle layer cells and the intermediate zone cells is visibly bright. In the superficial layer cells more condensed cytoplasm and usually a lack of nuclei were observed. The ultrastructural image of the basal layer cells and the basement membrane corresponded with the standards (Fig. 1). Normal ultrastructural images were also observed in other epithelium layers. In the keratinocytes of the prickle layer a large amount of the alpha and beta glycogen granules were found and also meagre filament bundles localized especially perinuclearly or around the cell membranes. Both smooth and rough endoplasmatic rete were quite moderate and sometimes revealed meagre widening. Desmosomes and non-desmosomal spaces were unchanged. Sometimes, scarce melanosomes were observed in the intracellular spaces, especially in the lower parts of the prickle and basal layers. Marked thickening of the cell membranes was stated in the superficial layers. Meagre cytoplasm of the keratinocytes of these layers contained a small amount of glycogen and was markedly thickened. Group II of the research consisted of the epithelium segments of the clinically unchanged oral vestibule mucosa frozen with the temperature of -96°C within 1 second collected directly after cryoapplication from the same 21 patients as in group I. It was found that on the semithin sections observed in the light microscope under the immersion with the magnification of 1000 times there was a widening of the intracellular spaces which was most intense at the basement membrane and decreasing towards the epithelium surface (Fig. 2). The creation of the perinuclear halo can be observed in the cells situated directly by the basement membrane. Although the appearance of this increased transparence around the nucleus

did not lead to the change in shape of the nuclei themselves, however, in the middle layers of the epithelium, at the distance of about 150 microns from the basement membrane, the creation of the larger perinuclear halo together with the simultaneous change in the shape of nuclei can be observed. By the surface of the epithelium the intracellular spaces decrease and the cells do not reveal any considerable deviation aberrations. In the site of adherence of the cryoprobe the border of the epithelial cells is blurred, there is a lack of nuclei and amorphous, intensively stained cytoplasm is visible.

Ultrastructural research reveals little or meagre widening of the intracellular spaces, especially in non-desmosomal spaces retaining regular desmosomes structure (Fig. 3), and the cell membrane does not break. Numerous clearings are visible in the cytoplasm of the cell, probably as a result of crystallization of intercellular water (Fig. 4). There are also numerous accumulations of filaments around desmosomes and dispersed in hyaloplasm (Fig. 4). These filaments sometimes created the bundles that were not very thick. Clearings of the matrix in mitochondria were not very large. The cytoplasm of some cells was visibly condensed. In such keratinocytes large accumulations of alpha and beta glycogen granules were observed. In many cells perinuclear halo appeared (Fig. 4) together with the simultaneous considerable deformation of the nuclei surfaces. Clearings of irregular shapes and different sizes are often visible within this perinuclear halo. They are probably the effect of the crystallization of intracellular water. The nuclei surrounded with clearings revealed irregular shape, marginal condensation of chromatin around the nuclear sheath, meagre euchromatin and electron thick nucleolus with the vague internal structure.

DISCUSSION

The introduction of low temperatures as a method of treatment was based on the following assumptions: evoking a limited range of the local necrosis, the possibility of bloodless separation of the diseased tissue, the healing process without any complications and the formation of a scar, which is thin, elastic, and satisfying in the cosmetic and functional respect. A very important advantage of cryotherapy is the possibility of association with the conventional surgical methods, chemotherapy, laser therapy. It can also be used in patients with malignant neoplasms who had the relapse after the radiological treatment (1). Cryosurgery is the method of choice in the treatment of benign neoplasms, preneoplastic conditions, preinvasive carcinoma and some non-malignant proliferative changes of the skin and mucous membranes. Moreover, it is used with satisfying results in the treatment of infections, bacterial, viral diseases of the skin and mucous membranes, Wilson's lichen and neuralgia, especially of the

trigeminal nerve (1, 4, 8, 10). According to Krwawicz, Szwarc and Lenkiewicz research (5, 6, 11) in ophthalmology after the application of cryotherapy fast progress of the regeneration and separation of tissue is observed. According to Szyszkowska research, both in the skin and tongue mucous membrane of rabbits, fast progress in the reconstructive reaction was stated and likewise the contribution of the hydrolytic tissue enzymes in the necrosis caused by freezing (12, 13, 14). The following results can be valuable information suggesting the pathomechanism of retrogressive changes observed in the epithelium and interstitium after the application of low temperatures. The quoted authors emphasize greater sensitivity of the tongue mucous membrane than the skin to the activity of similar freezing parameters. It is the confirmation of the information brought by many researchers (8) emphasizing tissue and organ peculiarity as an essential feature which conditions the range and character of the cryodestructive changes. Other essential parameters defining the degree of the destructive changes are: freezing temperature, surface and freezing time, tissue vascularization and the functional and morphological state of blood vessels (2, 3). Moreover, the formation of thrombi is observed in the thermally damaged vessels, causing secondary tissue hypoxia (3, 7, 10). After freezing for 1 second it was observed that there was a widening of the intracellular spaces and there were vacuoles in the keratinocytes of the basal and prickle layers. Moreover, it was stated that there was oedema of the mitochondria without breaking of the external sheath of these organelle.

REFERENCES

1. Adamus J. et al.: Kriochirurgia w leczeniu nowotworów jamy ustnej. Otolar. Pol., 28, 1, 41, 1974.
2. Ernst F. D. et al.: Various cytological foundations and consequences of cryosurgery. Z. Exp. Chir., 12, 3, 163, 1979.
3. Ernst F. D. et al.: Zur Kryobiologie der Mundhöhle. Dtsch. Stomatol., 21, 714, 1971.
4. Hausamen J. E.: The basis, technique and indication for cryosurgery in tumours of the oral cavity and face. J. Max-Fac. Surg., 3, 1, 41, 1975.
5. Krwawicz T.: Przeszłość, stan obecny i przyszłość kriooftalmologii. Kl. Ocz., 44, 325, 1974.

6. Lenkiewicz E.: Krioterapia doświadczalnych oparzeń spojówki gałkowej i rogówki. Kl. Ocz., 44, 15, 1974.
7. Levelock J. E.: The haemolysis of human red blood-cells by freezing and thawing. Biochem. Biophys. Acta, 10, 414, 1953.
8. Przywara S. et al.: Wstępna ocena wyników leczenia nowotworów skóry i śluzówek jamy ustnej metodą kriochirurgiczną. Pol. Prz. Chir., 48, 4, 487, 1976.
9. Rosen G. et al.: Cryosurgery for basal cell carcinoma of the head and neck. Sud. Afr. Med. J., 56, 1, 26, 1979.
10. Schargus G. et al.: Cryosurgical devitalization of bone and its regeneration. J. Max. Fac. Surg., 3, 2, 128, 1975.
11. Szyszkowska A.: Badania eksperymentalne wpływu niskiej temperatury na tkankę jako podstawa klinicznego stosowania kriochirurgii. Pr. dokt., Lublin 1981.
12. Szyszkowska A. et al.: An effect of low temperatures on the morphological picture of the rabbit tongue mucosa. Gegenb. Morph. Jahr., 129, 6, 707, 1983.
13. Szyszkowska A. M. et al.: Effect of low temperatures on the morphological picture of the rabbit skin. Gegenb. Morph. Jahr., 129, 6, 719, 1983.
14. Terracis L. et al.: Freezing and drying of biological tissues for electron microscopy. J. Histochem. Cytochem., 29, 9, 1021, 1981.

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STRESZCZENIE

Wieloletnie światowe badania doświadczalne i kliniczne nie wyjaśniły jednoznacznie mechanizmu działania niskich temperatur. W miarę rozszerzania wskazań do zabiegów kriochirurgicznych traktowanych jako metoda leczenia w różnych przypadkach chorób jamy ustnej i twarzy wyłania się potrzeba przewidzenia i zaplanowania wielkości obszaru podlegającego kriodestrukcji i określenia głębokości uszkodzenia. Celem pracy była ocena rodzaju zmian zachodzących w komórkach nabłonka niezmienionej klinicznie błony śluzowej jamy ustnej, przy zastosowaniu urządzenia kriochirurgicznego AUK-20A z zamkniętym aplikatorem o średnicy 10mm, oziębionym do temp. –196°C, stosując czas zamrażania 1 sek. Oceniano wycinki błony śluzowej jamy ustnej, pobrane od 21 pacjentów - w tym 10 mężczyzn w wieku od 28 do 76 lat i 11 kobiet w wieku od 17 do 67 lat. Pobrane wycinki

z miejsca przyłożenia kriosondy utrwalano w 4% aldehydzie glutarowym i 1% czterotlenku osmu oraz zatapiano w zestawie Spurr Low-Viscosity (Polysciences USA).

Preparaty półcienkie barwiono 1% Azurem II i 1% błękitem metylenowym w 1% boraksie. Preparaty ultracienkie dokontrastowywano octanem uranylu i cytrynianem ołowiu. Preparaty oceniano w mikroskopie świetlnym i elektronowym Tesla BS 500.

W obszarze przyłożenia kriosondy zmiany w komórkach nabłonka błony śluzowej jamy ustnej są najbardziej nasilone w okolicy błony podstawnej. Poszerzenie przestrzeni międzykomórkowych oraz tworzenie się wodniczek w cytoplazmie jest wynikiem krystalizacji wody wewnątrz i pozakomórkowej. Obserwowane zmiany w mitochondriach i cytoskeletonie należy rozważać jako uszkadzające i potencjalnie odwracalne.

EXPLANATION TO FIGURES

Fig. 1. The ultrastructural image of basal layer cells of the epithelium of the oral mucosa. Basal membrane (BM) uninterrupted with the regular electron density. The ultrastructural image of hemidesmosomes, anchoring fibrils, desmosomes and non-desmosomal intracellular spaces regular. Nuclei with not very abundant heterochromatin concentrated on the nuclei rims and quite abundant euchromatin. Moreover, present regular mitochondria (M) meagre endoplasmic reticulum (ER) and bundles of intermediary filaments (F). Mag. 6,000x.

Fig. 2. The cross-section of the epithelium of the oral mucosa in the site of the application of cryoprobe (temp. -196^{0}C, time 1 sec.). The basal layer cells lower strata of the prickle layer shrinked with considerable widening of the intracellular spaces. In the distance of about 150mm from the basement membrane visible perinuclear vacuoles with accompanying change of the nuclei shape. In the superficial layer blurring of the cell borders among keratinocytes, lack of nuclei and intensively staining cytoplasm of the keratinocytes. Mag. 14,000x.

Fig. 3. Slight widening of the non-desmosomal intracellular spaces retaining the continuity of cell membrane and normal image of the desmosomes. In the cytoplasm of cells present numerous alpha and beta glycogen granules and the accumulation of filaments usually localized around desmosomes. Intermediate epithelium layer. Mag. 14,000x.

Fig. 4. Slight widening in the intracellular spaces in the non-desmosomal spheres with normal desmosome image. Numerous vacuoles of medium thickness surrounding the nuclei. In some of these vacuoles clearings are observed. The keratinocytes nuclei of irregular shape, some of them shrinked. In the cytoplasm present numerous tiny vacuoles and the bundles of compact filaments. Epithelium prickle layer. Mag. 2,500x.

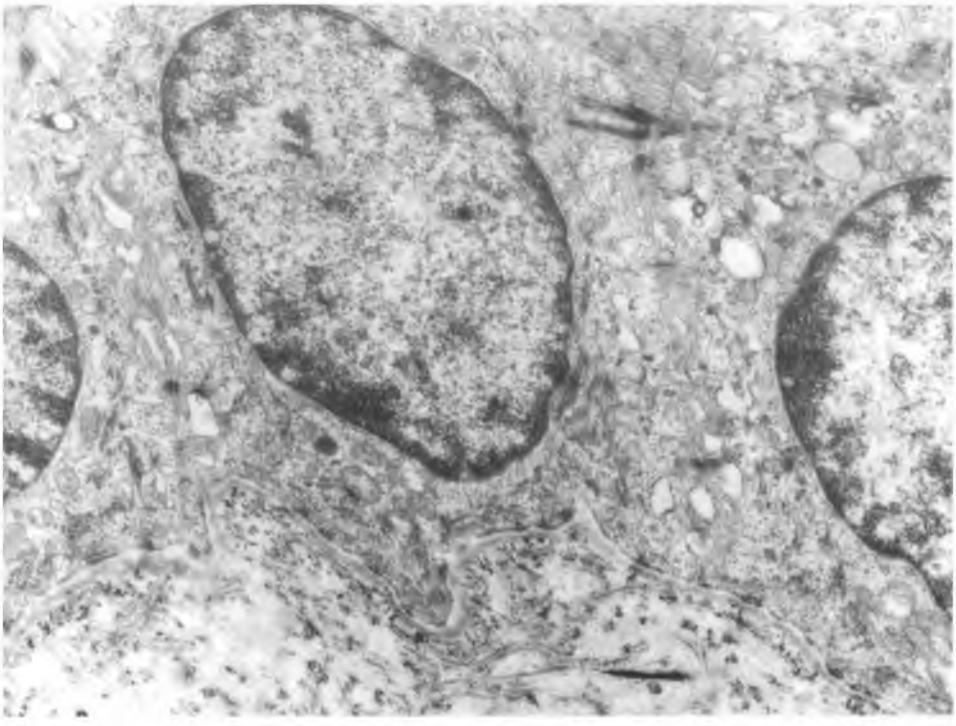


Fig. 1

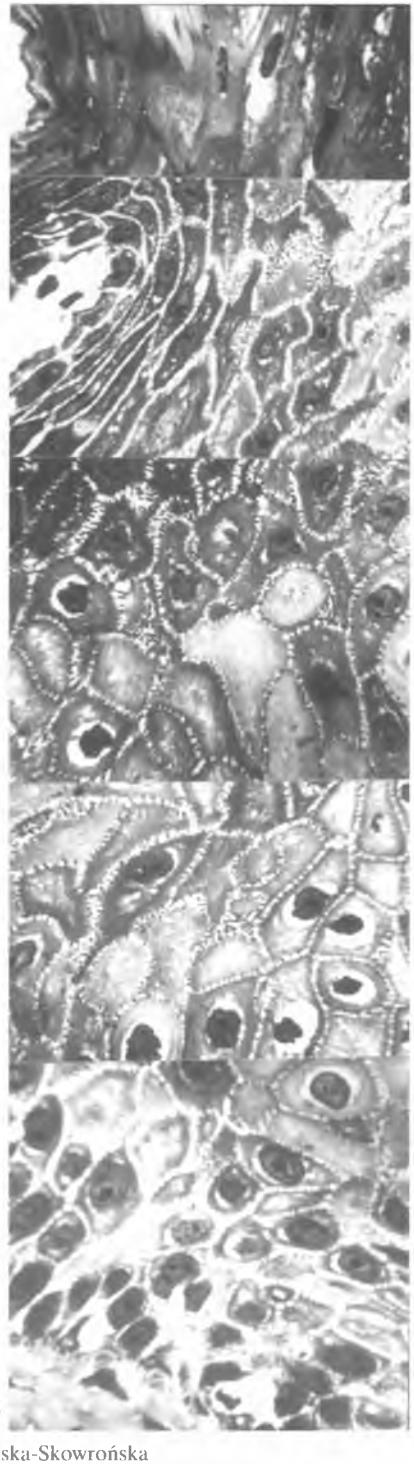


Fig. 2

E. Gałkowska, Z. Sieczeniewska-Skowrońska

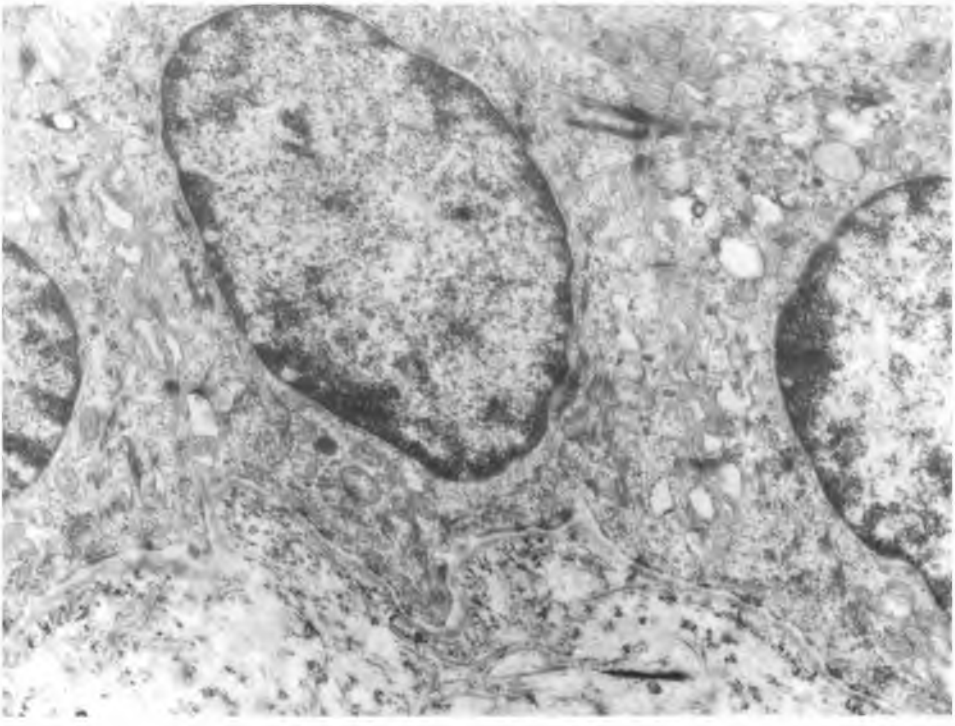


Fig. 1

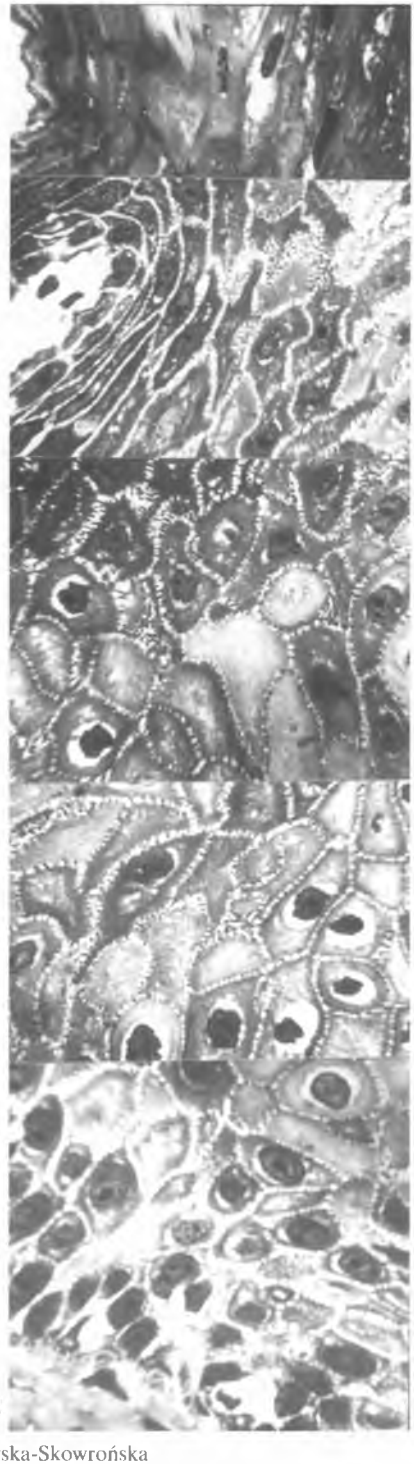


Fig. 2

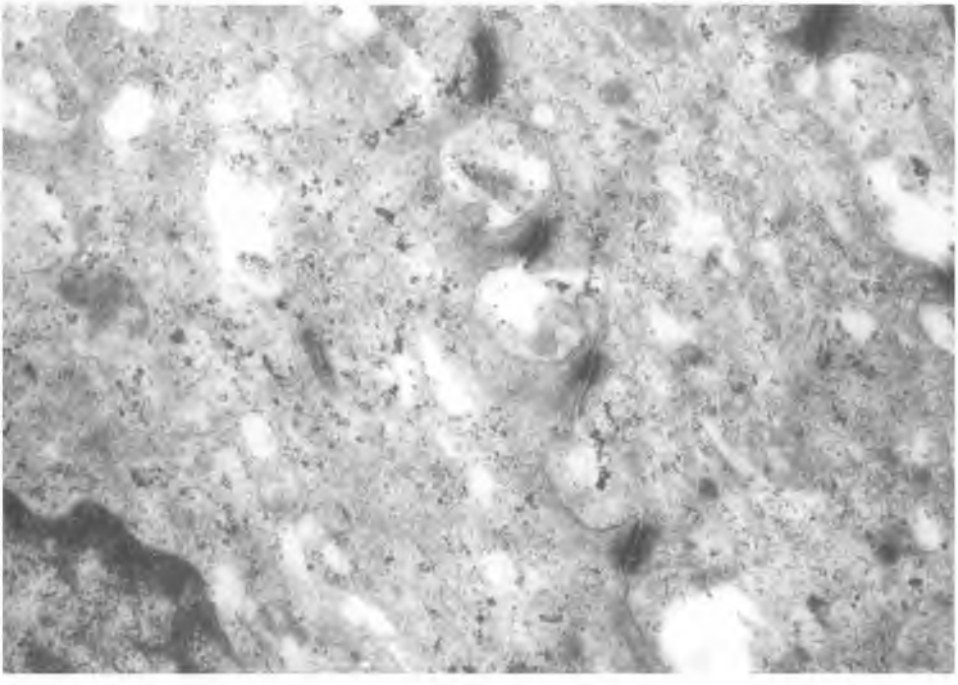
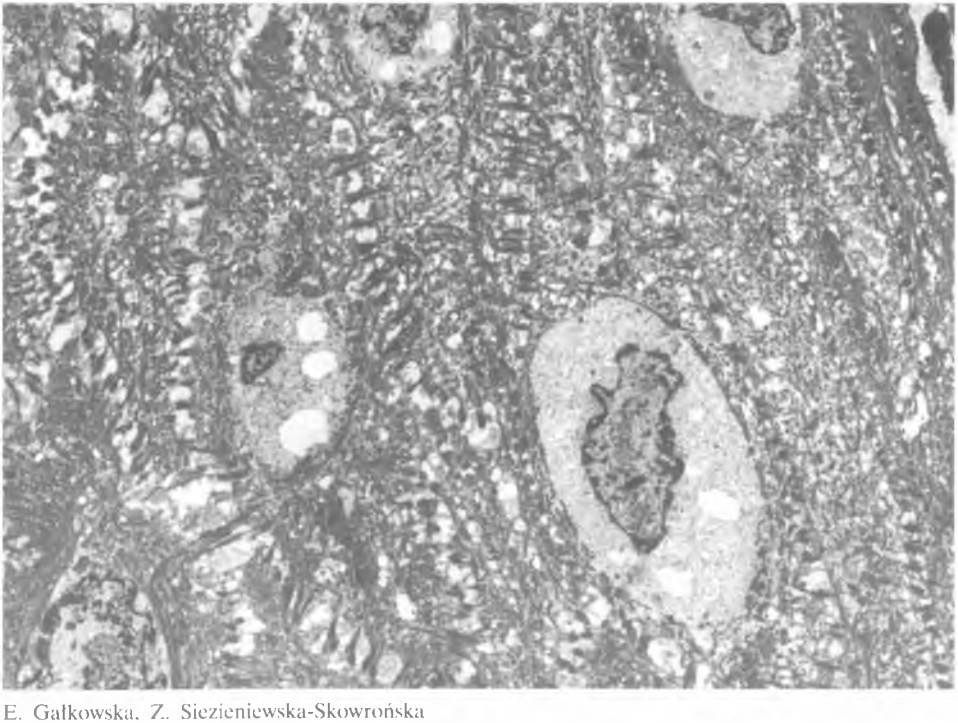


Fig. 3

Fig. 4



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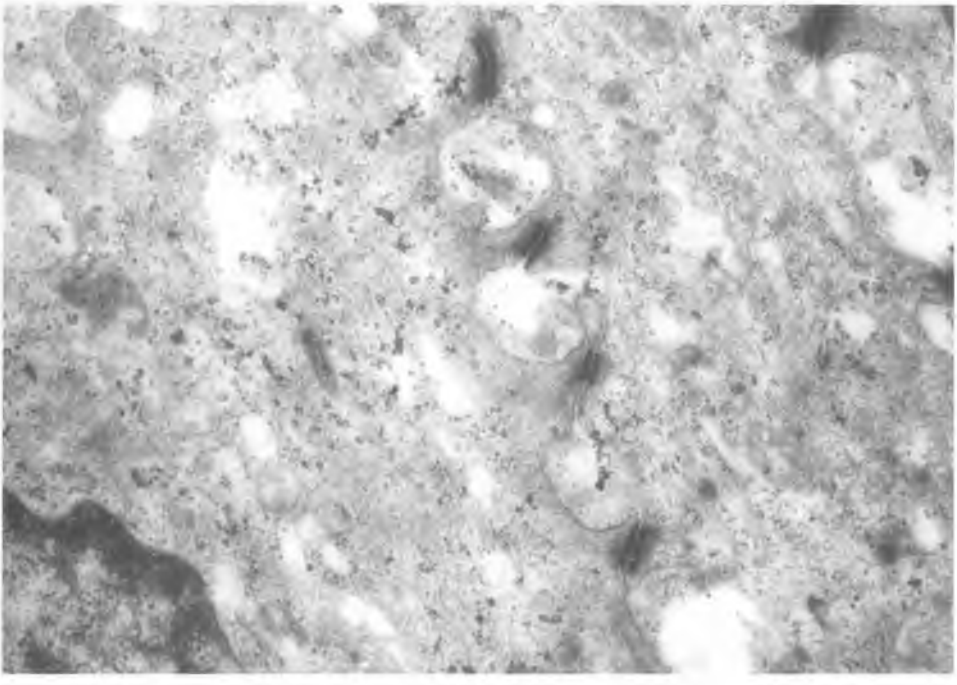
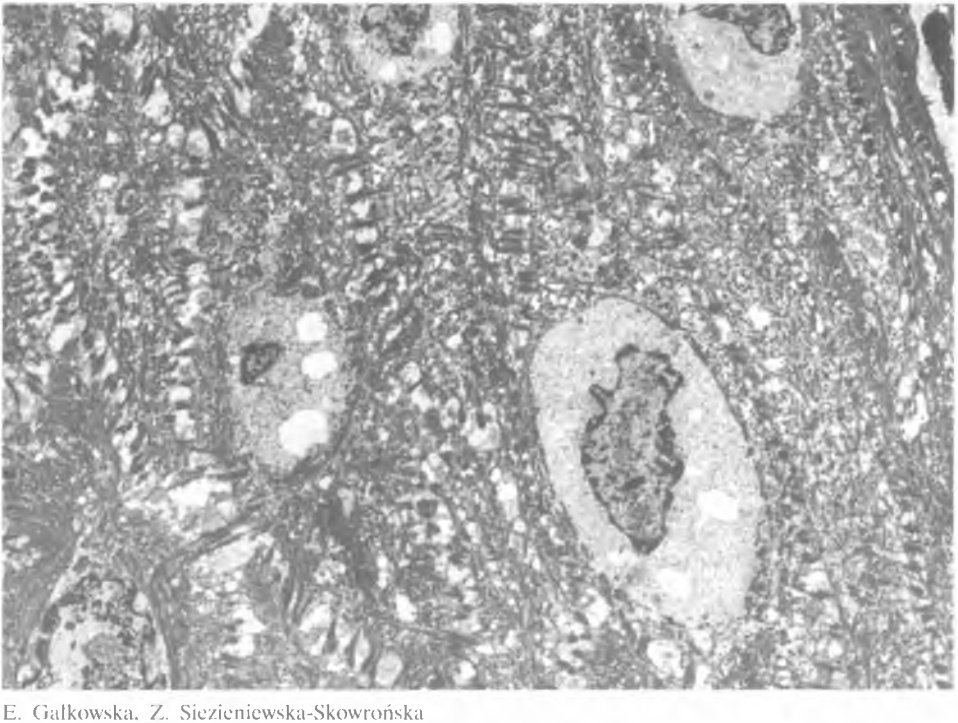


Fig. 3

Fig. 4



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