

Ultrastructural Changes of *Candida albicans* Species Induced by the Presence of Sodium Diclofenac

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The high frequency of occurrence of candidiasis as well as high mortality of patients with immunosuppression cause a tendency toward better understanding of Candida albicans species virulence factors and developing sensitive and specific diagnostic methods, and appropriate strategies of candidiasis treatment. In recent decades the incidence of fungal infections has alarming increases because of advanced medical treatments. In this study was analyzed possible ultrastructural changes of the species C. albicans cells following treatment with sodium diclofenac at various concentrations. Following treatment of C. albicans cells with sodium diclofenac 1 mM and 2 mM changes in the plasmalemma can be noticed, changes in the density of cell wall, disruption and necrotic appearance of the cytoplasm.

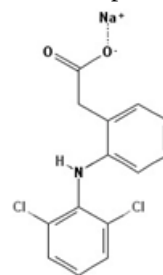
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Candida (C.) albicans is the most frequent cause of fungal infections. As a result of advanced medical treatments and the increase in the number of immune-compromised and severely ill patients, in recent decades the incidence of fungal infections has alarming increases [1].

Candida albicans is a dimorphic fungus that is able to adopt a wide range of morphologies; therefore, it is considered a *polymorphic* or *pleomorphic* microorganism. *Candida albicans* can reproduce itself by budding as yeast cells (blastospores or blastoconidia). Under certain growth conditions, *C. albicans* can form chlamydospores which have a circular shape and a thin cell wall. These morphological transitions represent the response of yeast cells to changes in environmental conditions and allow it to adapt to different biological niches. The ability of this species to adopt different morphologies is essential to infection dissemination.

Research carried out by different authors, which considered drug substances used for purposes other than fungal infections, noticed a number of changes at the ultrastructural level in the case of potentially pathogenic yeast *Candida albicans*. In this respect, there have been conducted ultrastructural analysis on cells from the species *C. albicans* subjected to treatment with ibuprofen, sodium caprylate and miconazole. In the case of yeast cells treated with ibuprofen, a concentration of 20 mg/mL produces changes in the cell membrane, having a solubilizing effect on it [2]. Significant changes occur at the level of *C. albicans* spheroplasts undergoing treatment with ibuprofen at a concentration of 12.5 mg/mL. Studies carried out in the presence of sodium caprylate at various concentrations (0.025-0.1 M) on *C. albicans* cells show condensation of the vacuole material and mitochondria changes with regard to their number and size, as well as increase of the cytoplasm electron density [3]. Research carried out in the presence of miconazole antifungal drug with concentrations of 10^{-4} M, highlights the ultrastructural changes that occur in the plasmalemma. It can also be noticed the necrotic appearance of the cytoplasm, lipid

deposits occurrence as well as vesicular formations (representing cytoplasmic material) in the vicinity of the cell wall [4]. A number of studies have considered the effect of saponin SC-2 (extracted from *Solanum chrysotrichum*), which has antifungal activity on various species of *Candida* (*albicans*, *glabrata*, *tropicalis* and *krusei*). Ultrastructural changes induced by the presence of saponins were: deterioration of the cytoplasmic membrane and organelles, changes in morphology and cell wall density, accompanied by the separation of the cytoplasmic membrane from the cell wall, as well as total degradation of cellular components [5].



Sodium diclofenac

In the literature we have not found data concerning the analyses conducted at the ultrastructural level regarding the action of sodium diclofenac on the *Candida albicans* species. Diclofenac and other non-steroidal anti-inflammatory drugs are widely used therapeutics, primarily for the treatment of pain and inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of all inflammatory diseases, and most of them exhibit analgesic and antipyretic properties. The diclofenac, [2-[(2,6-dichloro-phenyl) amino -phenyl] -acetic acid, is a NSAID used in inflammation and painful cases of rheumatic and anti-rheumatic origin. Mostly, it is sold as a sodium salt [6]. These drugs inhibit the cyclooxygenase isoenzymes (COX) COX-1 and COX-2, which are involved in the biosynthesis of mammalian prostaglandins. Prostaglandins (PG) are small lipid molecules with diverse

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biological functions. The mechanism of action of sodium diclofenac is not often fully known. It is known that its action anti-inflammatory, antipyretic and analgesic is due to the PG biosynthesis inhibition through COX inhibition. COX inhibition has the effect decrease of PG in gastric epithelial cells, thereby sensitizing the gastric mucosa to the corrosive gastric juice (the main side effect). Diclofenac has a moderate preference to block the COX-2 isoenzyme (approximately ten-fold as compared to COX-1) and, therefore, gastrointestinal side effects have a lower incidence in comparison with indomethacin or aspirin. Some studies suggested that PGs can be viewed as regulators of *C. albicans* virulence because the eicosanoids pathway in *C. albicans* species plays a central role in the control of morphogenesis and biofilm formation. As a consequence, they further suggested that the development of drugs that specifically target the fungal prostaglandins pathways may be one strategy to combat fungal colonization and infection [7].

The main purpose of this study was to analyze possible ultrastructural changes of the species *C. albicans* cells following treatment with sodium diclofenac at various concentrations.

Experimental part

Materials and methods

In order to identify the effects caused by the presence of diclofenac sodium on *C. albicans* strains, we analyzed under the electronic microscope, the yeast cells (one strain) treated with this non-steroidal anti-inflammatory substance. In order to achieve this experiment, we used a cell suspension *C. albicans* containing culture medium (YPG) sodium diclofenac in concentration of 1 mM and 2 mM. After 24 h incubation at 37°C, the cell sediment is subjected to the protocol for the analysis by electron microscopy. The standard method of electron microscopy was used, adapted to the requirements of our biological material [8]. The steps were as follows: prefix in 3% glutaraldehyde in cacodylate buffer at + 4°C overnight; fixation in 2% osmium tetroxide solution at + 4°C overnight; successive washes with distilled water; subsequent dehydration in ethyl alcohol in order in increasing order of alcohol concentrations: 10, 30, 50, 70, 100%, mixture of 100% alcohol - propylene oxide of 1:1, and pure propylene oxide; infiltrating the resin EPON 812; the polymerization at temperatures of 40 and 60°C for 48 h; ultramicrotome ultra sectioning. The sections were made using a diamond cutter in a LKB Ultratome. Their contrasting was performed with uranyl acetate and lead citrate according to Reynolds method [9]. Examination of sections was done under an electron microscope EM-125 (SEIMI- Ukraine).

For this study we used: medium YPG-(Yeast Peptone Glucose- 1% yeast extract, 2% glucose, 1% peptone), phosphate buffer with pH=7.4, and stock solution of the sodium diclofenac – 100 mM (in ethanol and phosphate buffer).

Results and discussions

In this study, electronic microscopy observations were utilized to investigate the anticandidal activity of sodium diclofenac. The microscopic examination of *C. albicans* strain showed that the cells treated with sodium diclofenac appeared irregular in shape with cell wall modification, ruptured cell membranes. Electronmicroscopic images obtained by the analysis of the *C. albicans* cells reveal a typical pattern of the yeast cells. They highlight the mitochondria, nucleus, electronodense cytoplasm bodies (probably glycogen granules) inside an electronodense

cytoplasm. Cell wall thickness varies with the age of the cell (it increases as the cell grows older).

Untreated *C. albicans* cells (control: figs. 1 and 2) present nucleus, mitochondria and slight invagination of the plasma membrane that become more pronounced as they are treated with sodium diclofenac 1 mM and 2 mM (fig. 4). Treatment with 1 mM diclofenac caused yeast cell ultrastructural changes, translated in increased content lysis of cytoplasmic structures which gives the appearance of a *rarefied* structure caused by vacuole degradation and release its lytic contents (figs. 3 and 4). Folding of the plasmalemma become apparent as well as its disruption; also, there can be noticed numerous glycogen granules, bringing together features of senescent or necrotic cells. Membranes appeared disrupted and organelles such mitochondria and nucleus lost conventional morphology. Damage increased gradually, with the disappearance of organelle organization, cytoplasm degradation and finally, cellular disintegration.

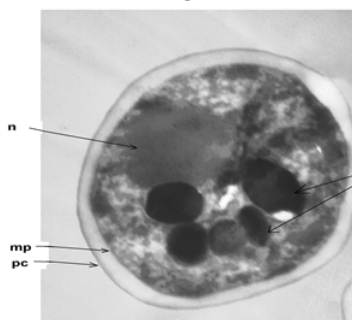


Fig. 1. *C. albicans* untreated cell: n-nucleus; g-glycogen granules; pc-cell wall with regular contours and electronodense outer layer; mp-plasma membrane (x25000)

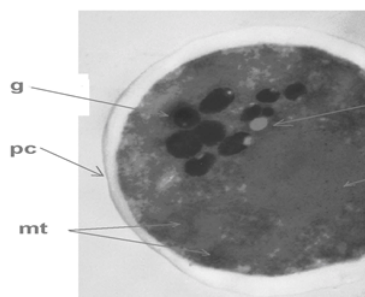


Fig.2. *C. albicans* untreated cell: n-nucleus; g- glycogen granules; pc-cell wall with regular contours and electronodense outer layer; c.l.- lipid bodies; mt-mitochondria; there is an electronodense cytoplasm (x25000)

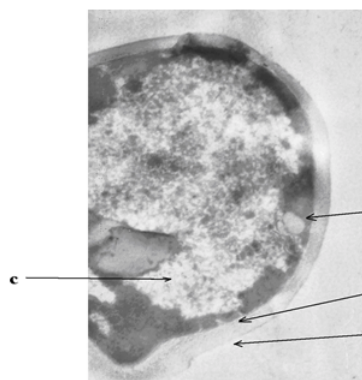


Fig.3. *C. albicans* cell treated with 1 mM diclofenac: pc-electronotransparent outer layer cell wall; c- necrotic appearance of the cytoplasm; mp-plasma membrane has invaginations; c.l.- lipid bodies nearby plasmalemma (x25000)

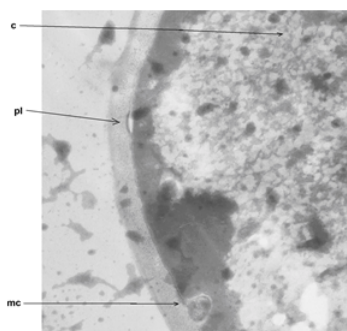


Fig.4. *C. albicans* cells treated with 1 mM diclofenac: c-necrotic cytoplasm; pl-detachment of plasmalemma from the cytoplasm; mc-cytoplasmic material (x37000)

In ultra microphotographs it was highlighted the particular aspect of plasmalemma showing pronounced folding, stronger than in cells grown in the absence of sodium diclofenac (fig. 5). This is due to the involvement of plasmalemma in intense metabolic activities, mainly related to cell wall biogenesis. Moreover, data from the literature based on electron microscopy studies have shown plasmalemma role in the regeneration of the cell wall [10]. Thus, it has been shown that the first stage of regeneration to form a network composed of fibrillar glucan and crystallized chitin. Glucan and chitin are polymerized by synthases located in plasmalemma. The agar media, the matrix component appears at 30-40 min after the fibrillar network structure of the polysaccharide. Chitin and β -1,3 glucans are synthesized in the plasma membranes and released in the periplasm, mannoproteins are synthesized in the cytoplasm and transported by secreting routes.

Plasma membrane represents a significant barrier actively participating in various cellular processes at the cell interface level, such as reactions to the extracellular medium, morphogenesis and cell wall biogenesis. In addition, the vast majority of pharmaceutical drugs, including antifungal medication affect the plasma membrane components. Although it is of particular importance in the structure, morphology and physiology of yeast cell, the intimate structure of the plasma membrane is not fully deciphered, mainly because of technical limitations and its hydrophobic character [11].

In general, the cell wall of yeast is about 15-30% of the whole cell weight and has a size between 0.2 and 0.3 μ m [12]. Cell wall of *C. albicans* species consists mainly of mannoproteins and glucans. The cell wall is electron-transparent except the outer layer which is electron-dense; the thickness of the external wall is 0.05-0.1 μ m [3]. In the case of cells treated with 1 mM sodium diclofenac it can be observed that the outer layer of the cell wall becomes less electron-dense (figs. 3, 4, 5 and 6).

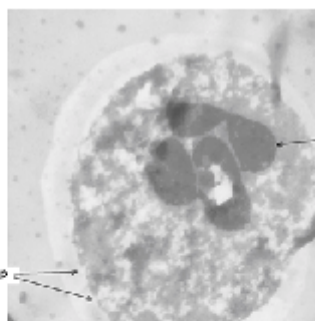


Fig.5. *C. albicans* cells treated with 1 mM diclofenac: cytoplasm; cw-cell wall; g- glycogen granules; mp-plasmalemma folding (x26000)

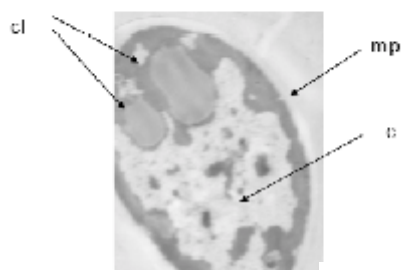


Fig.6. *C. albicans* cell treated with diclofenac sodium 2 mM: cl-lipid bodies; c- cytoplasm with necrotic appearance; mp-folding of plasmalemma; it can be remarked the fringes aspect of cytoplasm (x22500)

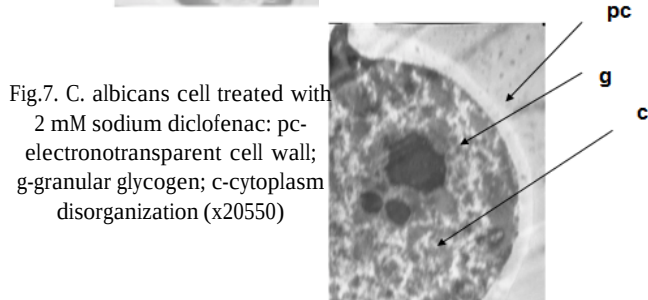


Fig.7. *C. albicans* cell treated with 2 mM sodium diclofenac: pc-electrontransparent cell wall; g-granular glycogen; c-cytoplasm disorganization (x20550)

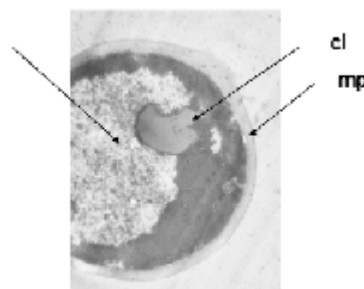


Fig.8. *C. albicans* cell treated with 2 mM sodium diclofenac: mp-separation of the cytoplasm from the plasmalemma; c-necrotic cytoplasm; cl-lipid bodies (x23400)

It also can be observed that the cell wall becomes thinner and the reduction of its thickness is enhanced by increasing the concentration of diclofenac (figs. 7 and 8). The cell wall has a regular shape while gradually plasmalemma is changed.

Diclofenac concentrations of 2 mM induce almost complete disruption of cytoplasmic contents. Cytoplasm, which in control cells was electron-dense, in this case appears completely dispersed into smaller fragments. They are pushed towards the plasma membrane and stored on the internal face of it. Cells become necrotic. In figure 4 it can be observed the cytoplasmic material loss following damage of the cell wall and plasma membrane disruption; plasmalemma detachment from the cytoplasm is highlighted. The cytoplasmic membranes of yeast is a barrier to passage of small ions such as K^+ , Na^+ , Ca^{2+} , H^+ and allows cells and organelles to control the entry and exit of different compounds. This permeability barrier role of cell membranes is integral to many cellular functions, including the maintenance of the energy status of the cell, other membrane-coupled energy-transducing process, and metabolism regulation [13].

All these factors have resulted in an aggressive oxidative stress that is expressed through the release of oxygen free radicals (ROS), mitochondria being the first affected. ROS, normally produced during the aerobic metabolism, function as second messengers involved in many cellular functions. In fact, ROS may cause severe damages to several cellular macromolecules, including proteins, lipids, and DNA, thus contributing to the development of many pathological conditions.

Mitochondria are cellular organelles, bounded by membranes, which are self-replicating. They are found in variable number, shape and size in eukaryotic cells. Mitochondria is the place where the electron transport chain and oxidative phosphorylation leads to regeneration of ATP. There are oxidative phenomena occurring at the cell membrane level by degrading lipoprotein membranes and chromatin and nucleus modifications. Thus, by treatment with sodium diclofenac mechanisms of induction of oxidative stress that results in degradation of the *Candida albicans* cell may occur.

Our results demonstrate changes at ultrastructural level induced by the presence of sodium diclofenac on the species *Candida albicans*: degradation of cytoplasmic membrane and organelles, changes in the cell wall density, accompanied by cytoplasmic membrane separation from the cell wall.

Previous research on the effect of anti-inflammatory drugs (aspirin, diclofenac) showed the inhibitory effect of these chemicals on cell viability and morphology of some *Candida albicans* strains [14, 15] and also diclofenac was able to induce direct mitochondrial dysfunction [16, 17].

Sodium diclofenac is a medication within the category of non-steroidal anti-inflammatory drugs. Scientific research has revealed that it exerts inhibition of lipoxygenase, thus reducing the formation of leukotrienes [18]. Moreover,

diclofenac can achieve an inhibition of phospholipase A₂, as part of mechanism of action. Phospholipases A₂ and B have been identified in a large number of pathogenic eukaryotic organisms such as the species *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. These phospholipases catalyze the cleavage of fatty acids chains in the phospholipid structure. They are considered as virulence factors of many pathogenic species, including *C. albicans*, by destroying the cell membrane of the host cell [19]. These collateral mechanisms could explain the increased potential of diclofenac, the most widely used drug in its category and the most visible effects. Except inhibition at COX isoenzymes as therapeutic target, there have been identified other targets of action for diclofenac: blocking the channels of sodium (diclofenac inhibits their reactivation), blocking ion acid-sensitive channels and it is a positive allosteric modulator of potassium channels (opens these channels, resulting in hyperpolarization of the cell membrane).

In patients who develop infections with *C. albicans* species occurs most often some resistance to this species to antifungal drugs, especially fluconazole. Furthermore, in patients with conditions which lead to the decrease of the immune system the biofilm produced by this species frequently develops. The antimicrobial activity of some anti-inflammatory drugs (diclofenac, ibuprofen) was investigated and results have shown that sodium diclofenac and ibuprofen in association with fluconazole and ketoconazole induced growth inhibition of *C. albicans* strains. Combination of non-steroidal antiinflammatory drugs (diclofenac and ibuprofen) with fluconazole or ketoconazole presented a synergic effect against *C. albicans* strains cell viability, inducing a decrease of cellular viability with 20-30% [20, 21]. The most common substances that are used for their antifungal actions are imidazole and triazole derivatives [22].

Conclusions

Using electron microscopy methods allowed, for the first time, revealing *Candida albicans* changes at the cellular level induced by the presence of sodium diclofenac, which is a non-steroidal anti-inflammatory drug substance and it is not recommended for the treatment of fungal infections. Following treatment of *C. albicans* cells with sodium diclofenac 1 mM and 2 mM changes in the plasmalemma can be noticed, changes in the density of cell wall, disruption and necrotic appearance of the cytoplasm. Exposure to diclofenac sodium caused cytoplasmatic membrane invaginations with marked structural disorganization of cytoplasm.

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