SUBMICROSCOPIC CHANGES IN THE PRODUCTION OF HYDROGEN PEROXIDE IN NECROTIC AND APOPTOTIC CELLS OF THE MOUSE POSTOVULATORY CUMULUS OOPHORUS

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A b s t r a c t

Numerous studies have recently provided evidence suggesting an association of cell injury and cell death with reactive oxygen species (ROS). In this study, the cytochemical cerium chloride procedure was used to detect *in situ* production of hydrogen peroxide (H_2O_2) in the mouse cumulus oophorus surrounding a postovulatory oocyte. Normal, active cumulus cells showed no presence of H_2O_2. However, H_2O_2 was regularly found in cells exhibiting ultrastructural signs of damage or death. The generation of H_2O_2 was observed in very early stages of necrotic or apoptotic processes characterised by minor ultrastructural alterations of the cell and/or cellular organelles. These events always preceded nuclear changes associated with cell death. Two different primary sites of H_2O_2 production were detected, i.e., the plasma membrane in necrotic cells and mitochondria in apoptotic cells. In the late phases of necrosis, H_2O_2 was demonstrated also on outer mitochondrial membranes and, in the late phases of apoptosis, it was also found on cell surfaces. With progression of apoptosis, multiple sites of H_2O_2 generation were detected in the cytoplasm, the nucleus or its fragments and in apoptotic bodies. A conspicuous finding was the presence of pairs of dying cells; in most of these, one cell was necrotic and the other apoptotic and between them a flow of H_2O_2 was detected. This suggests that H_2O_2 released by one of the cells may, like exogenous H_2O_2, act as a chemical signal inducing the onset of the dying process in its neighbouring cell.

K e y   w o r d s

Mouse cumulus oophorus, Ultrastructural detection, H_2O_2, Necrosis, Apoptosis

INTRODUCTION

Mouse postovulatory cumulus oophorus-oocyte complexes (COOCs), containing healthy and mature oocytes, are generally surrounded by ultrastructurally normal and functionally active cumulus cells (*1*). However, some cells exhibiting morphological signs of cell injury and/or necrotic and apoptotic cell death are also found (*2*).

Morphological features of dying cells have been studied extensively in recent years and are described elsewhere. Apoptotic cell death is characterised by cell shrinkage, cytosolic and nuclear condensation, plasma membrane blebbing, breakdown of nuclear DNA and, finally, separation of apoptotic bodies. Necrosis,

on the other hand, involves swelling and rounding-up of cells, loss of cell membrane integrity and spilling of cell content into the surrounding environment; cytoplasm disintegration is accompanied by the occurrence of small, irregular clumps of condensed chromatin in the nucleus.

Some recent data have shown that both necrosis and apoptosis are associated with reactive oxygen species (ROS) and that the most toxic intermediate is hydrogen peroxide (H_2O_2) because of its ability to cross membranes freely and to inhibit enzyme activities and cellular functions (*3,4*). Based on the possibility to detect H_2O_2 ultracytochemically *in situ* (*5,6*), the aim of this study was to prove it in a mouse cumulus cell population. The cumulus mass appears to be a very suitable model for such a study because it presents an avascular tissue containing a great number of individual cells, without any direct contact, embedded into the voluminous extracellular matrix. Therefore, in the cumulus oophorus mass, conditions for induction of an inflammatory response are absent.

MATERIALS AND METHODS

Mouse COOCs were obtained by flushing out the oviducts of virgin female mice (CBL/10 x CBA) F1 13 h after an injection of human chorionic gonadotrophine; COOCs were subsequently prefixed in a glutaraldehyde solution (300 mmol/l) in cacodylate buffer (100 mmol/l) for 30 min. For ultracytochemical detection of H_2O_2, individual samples were incubated in a medium containing cerium chloride (3 mmol/l) and 50 mmol/l sodium azide (inhibitor of endogenous peroxidase activity) according to *Briggs et al. (5)* for 30 min. The results of the cytochemical reaction were verified by the incubation of control COOCs either without sodium azide or with 2000 U/ml catalase (EC 1.11.1.6., Sigma, Germany) added to the incubation medium.

After the incubation of 45 COOCs in complete cerium media and of 38 COOCs in control media, all the samples were washed in cacodylate buffer (100 mmol/l) and postfixed in osmium tetroxide solution (40 mmol/l) in cacodylate buffer (100 mmol/l). They were then dehydrated and embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate solutions.

RESULTS

The results of our study showed that it was possible to detect H_2O_2 in a thoroughly fixed tissue. The sites where H_2O_2 was present were visualised by the formation of electron dense cerium perhydroxide precipitates in all samples incubated in the complete cerium media. On the other hand, no cerium perhydroxide precipitates, or only negligible traces of the reaction product, were found in the samples incubated either in the medium without sodium azide or in the presence of exogenous catalase.

In the cumulus cell population that surrounded an unaltered, mature mouse oocyte, there was a prevalence of morphologically intact cells in which H_2O_2 failed to be detected *Fig. 1)*. However, H_2O_2 was regularly detected in a low number of cells exhibiting some ultrastructural signs either of cell damage or cell death due to necrosis or apoptosis.
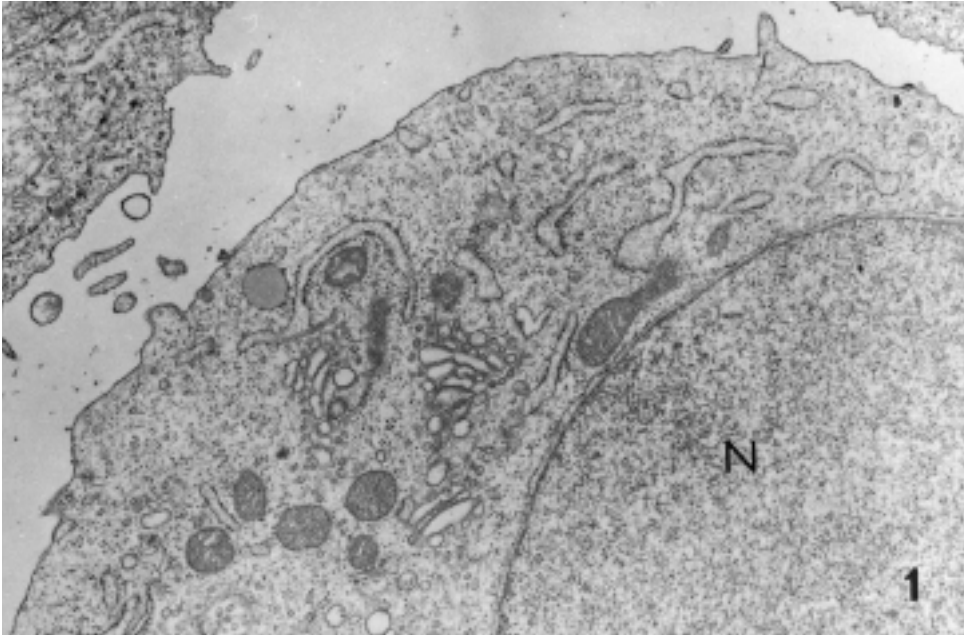
In the cells with mild ultrastructural features of the beginning necrosis manifested by a local swelling of the ground cytoplasm, cerium perhydroxide precipitates were primarily localised in limited cell surface regions covering the damaged cytoplasm. In cases of the total cytoplasm swelling accompanied by major plasma membrane defects, cerium perhydroxide precipitates covered the surfaces of injured cells completely *(Figs 2 and 3)*. In later stages of cell necrosis, H_2O_2 was detected also on the outer mitochondrial membranes *(Fig. 4)* of both normal and ultrastructurally altered mitochondria.

In cumulus cells that had continuous cell membranes but an increased density of cytoplasm, which is regarded as an early morphological sign of the onset of apoptosis, mitochondria were the primary sites of H_2O_2 production *(Fig. 5)*. In most of these mitochondria, the submicroscopic structure was damaged, but mitochondria of normal ultrastructural appearance were also observed. In both normal and injured mitochondria, cerium perhydroxide precipitates were bound, in the form of distinct granules, to the cytoplasmic side of the outer mitochondrial membrane. In the cells with ultrastructural signs of more advanced apoptosis, cerium perhydroxide precipitates also appeared on the plasma membrane *(Fig. 6)*.
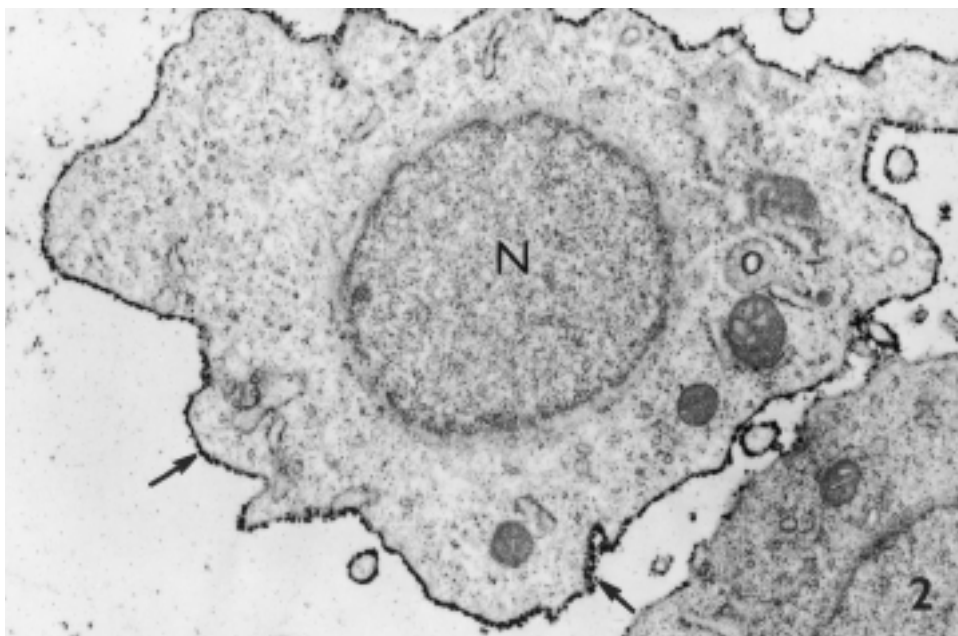
The cells in late stages of apoptosis were characterised by the occurrence of cerium perhydroxide precipitates also in the nucleus or its fragments, in the cytoplasm or apoptotic bodies *(Fig. 7)*. Hydrogen peroxide production usually decreased in the end stages of apoptosis and, in dead cells, cerium perhydroxide precipitates were no longer found *(Fig. 8)*.

Generation of H_2O_2 on the cell surfaces of both necrotically and apoptotically dying cells was generally accompanied by the release and penetration of this metabolite into the intercellular matrix *(Figs 3, 4, 6 and 9)*, often far from its cellular sources. The pairs of neighbouring cumulus cells, one with necrotic and the other with apoptotic ultrastructural characteristics, were often observed. Both cells showed H_2O_2 production on adjacent areas of their plasma membranes, and a flow of H_2O_2 was demonstrated between them *(Figs 4 and 9)*.

DISCUSSION

The presented results clearly documented an association of H_2O_2 production with ultrastructural signs of cell damage and cell death in mouse postovulatory cumuli oophori, because the presence of H_2O_2 was not detected in
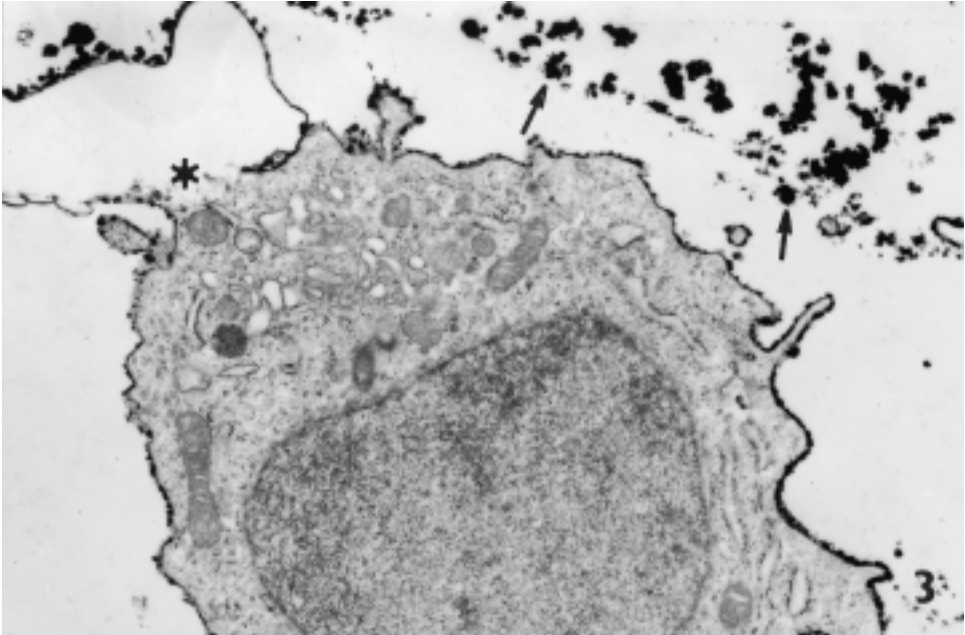
Fig. 3.
A necrotic cumulus cell with a discontinuous cell membrane (asterisk). Cerium perhydroxide precipitates cover the whole surface of the cell including the detached cell membrane fragment. A flow of H_2O_2 is visible in intercellular matrix (arrows). x 18,000.

morphologically intact, healthy cells. The negative results of this cytochemical reaction obtained in the healthy cells provide indirect evidence suggesting the existence of an effective control of ROS production by the endogenous intracellular antioxidant system, as proposed by *Kehrer (7).*

On the other hand, all the cumulus cells exhibiting some ultrastructural signs typical of both necrosis and apoptosis were capable of producing cytochemically detectable amounts of H_2O_2 at various phases of their dying. Hydrogen peroxide generation was evidently a very early event in cell damage; it was detected in the cumulus cells exhibiting only minute morphological alterations in the cytoplasm and cell organelles, while the nucleus retained a normal ultrastructural appearance.

In contrast to some literature data indicating that necrosis may be triggered by mitochondrial dysfunction and excessive ROS production (*8, 9, 10, 11, 12*), in this study, the first site of H_2O_2 production in the cumulus cells undergoing necrosis was the plasma membrane. The local H_2O_2 production was first demonstrated in limited areas of the plasma membrane covering the cytoplasm above small islands of swollen ground cytoplasm. With the progression of cell swelling and loss of cell membrane integrity, cerium perhydroxide precipitates became spread over the
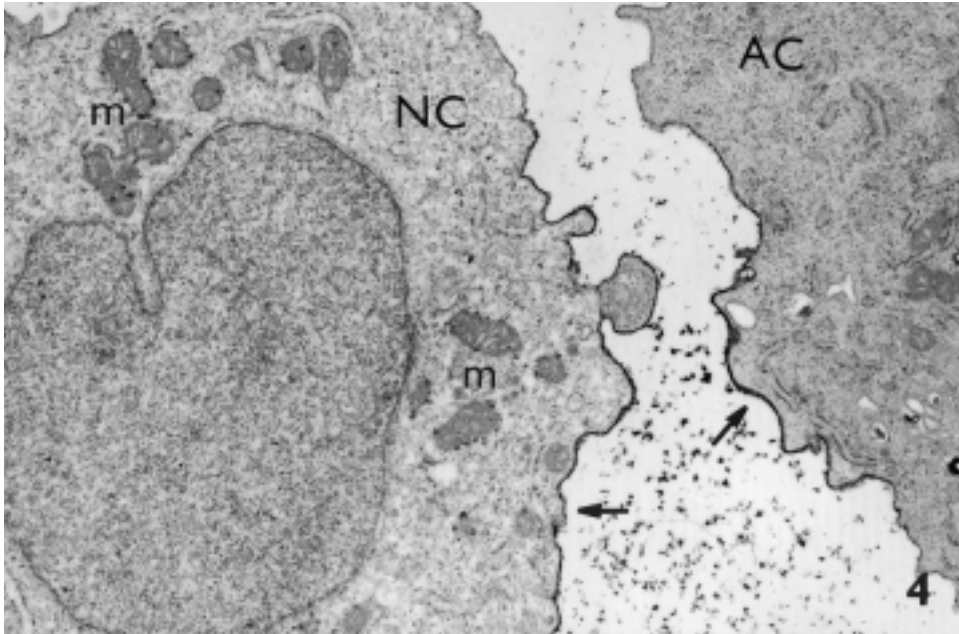
Fig. 4
A light necrotic (NC) and a dark apoptotic cell (AC). Note cerium perhydroxide precipitates in the adjacent regions of cell membranes and in the intercellular space between them (arrows). Mitochondria (m) with a reaction product on the outer membrane are visible in the necrotic cell exhibiting also a reduction in cell organelle number (compare with *Fig. 1*). x 15,000.

entire cell surface. These findings suggest that permeabilisation of the plasma membrane may, rather than mitochondrial dysfunction, be one of the earliest events in cell necrosis. In this study, the participation of mitochondria in H_2O_2 production was also demonstrated, but it was found in cells characterised by more advanced necrotic changes, such as damaged cell organelles and their lower numbers, and a sporadic appearance of small irregular clumps of condensed chromatin in the nucleus.

In many cumulus cells in the initial stages of apoptosis, the first activated sites of H_2O_2 production were mitochondria. Similarly to the cytochemical findings made in rat hepatocytes (*13*), cerium perhydroxide precipitates were found exclusively on the outer mitochondrial membranes of cumulus cells. The key role of mitochondria in the apoptotic cascade has generally been accepted. Superoxide anions of mitochondrial origin and their reactive oxygen species by-products (including H_2O_2) are supposed to cause mitochondrial membrane permeabilisation concurrent with a drop in ATP level and a decrease in cell respiration, which results in cell death (*10, 11, 12, 13, 14, 15, 16, 17, 18, 19*). In our study, the H_2O_2 production and ultrastructural alterations occurring in mitochondria regularly preceded chromatin condensation in the nuclei of apoptotically dying cumulus
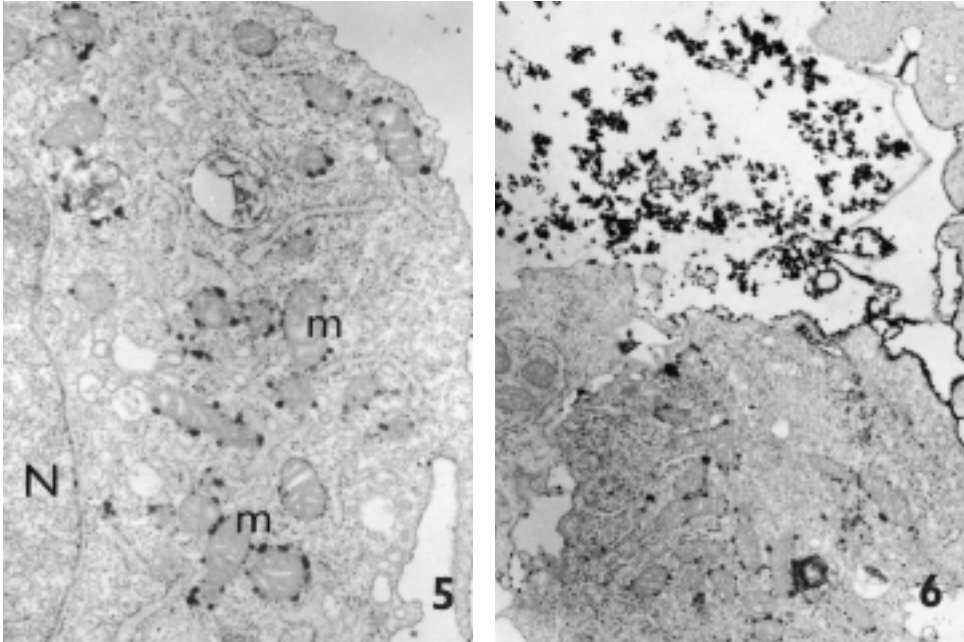
Fig. 5
An apoptotic cumulus cell with a continuous cell membrane. Hydrogen peroxide is detected on the outer mitochondrial membranes (m). Nucleus of normal morphological appearance (N). x 22,000.

Fig. 6
An apoptotic cell. Note cerium perhydroxide precipitates on the mitochondria, cell membrane, and in intercellular matrix. x 8,500.

cells. According to some data (*8, 20*) the apoptogenic protein released from mitochondria may act as a primary, apoptosis-inducing mechanism.

However, in some cells showing an increase in electron density and, therefore, being in the initial phase of apoptosis, the sole site of H_2O_2 localisation were not mitochondria but the cell membrane. Each such cell was regularly found in the close vicinity of another cumulus cell exhibiting some necrotic characteristics. The detection of H_2O_2 on the adjacent surface areas of these cells and in the intercellular space between them strongly suggests that H_2O_2 produced and released by one cell may act as a chemical death signal for the other cell. In view of the fact that both necrosis and apoptosis may be induced by exogenous H_2O_2 (*9, 11, 21, 22, 23*), it is difficult to distinguish which of these two cells acted as
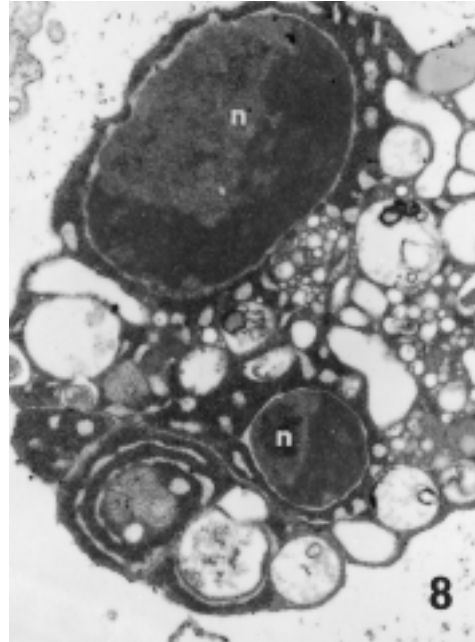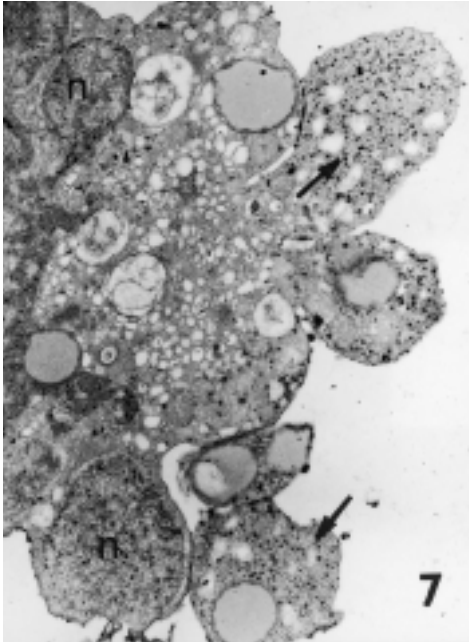
Fig. 7
A cumulus cell at the late stage of apoptosis. Cerium perhydroxide precipitates are seen in both the cytoplasm of developing apoptotic bodies (arrows) and some nuclear fragments (n). x 11,000.

Fig. 8
A cumulus cell at the final stage of apoptosis. Nuclear fragments (n). Cerium perhydroxide precipitates are no more visible. x 17,000.

a primary source of H_2O_2. Our results seem to indicate that necrotic cells are the cells inducing apoptosis in neighbouring cells, since H_2O_2 produced primarily on their surfaces rapidly penetrated into the surrounding environment. This assumption is also supported by the finding that necrotic oocytes producing and emitting H_2O_2 may induce a suicide programme in the entire cumulus cell population (2). However, the apoptosis of neighbouring cells may also be triggered via H_2O_2 generated by a primary apoptotic cell (24).

In addition to mitochondria and the plasma membrane, multiple sites of H_2O_2 occurrence were detected in cumulus cells at later stages of apoptosis. Cerium perhydroxide precipitates were found scattered in the cytoplasm of cells showing a reduction in cell organelle number, blebbing of the cytoplasm and separation of apoptotic bodies. A dispersed localisation of H_2O_2 was also typical of the nuclei that exhibited advanced chromatin condensation and sometimes nuclear fragmentation. This H_2O_2 dispersed localisation in the cytoplasm and nucleus may indicate that many intracellular macromolecules in the late stages of apoptosis are subject to oxidative damage.
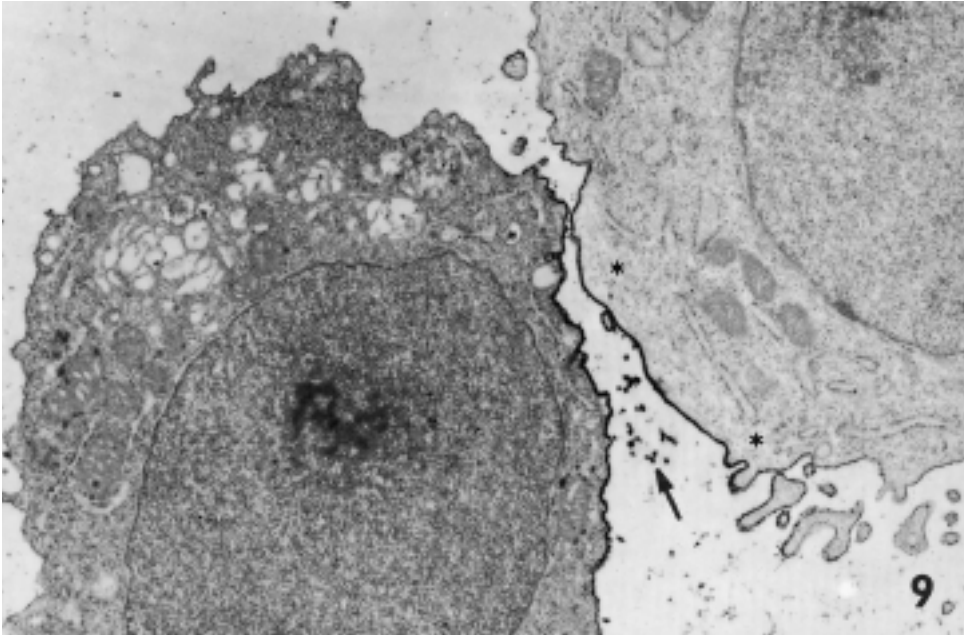
Fig. 9
A pair of neighbouring cumulus cells showing a positive reaction on the adjacent surface areas and in the intercellular matrix (arrow). The light cell shows an inconspicuous local swelling of ground cytoplasm (asterisk) and is considered to be at the initial phase of necrosis.The dark apoptotic cell possesses damaged cell organelles. x 15,000.

In conclusion, some considerations should be mentioned regarding the cytochemical detection of H_2O_2 in mouse postovulatory COOCs. In contrast to the results of *Briggs et al.* (5) and *Karnovsky (6)* who detected oxidase-caused H_2O_2 generation *in situ* only in unfixed or very lightly fixed cells and tissues, electron microscopic observations in this study clearly showed the possibility of H_2O_2 detection in samples thoroughly fixed with glutaraldehyde solution. The specificity of the used cytochemical reaction was verified by the absence of cerium perhydroxide precipitates after the addition of exogenous catalase (which uses H_2O_2 as a substrate) into the incubation medium. The cerium chloride incubation medium without the addition of sodium azide, which acts as an inhibitor of catalase and some other enzymes and probably also as a scavenger of superoxide anions *(25)*, substantially reduced or completely blocked the formation of cerium perhydroxide precipitates. The negative results of this control test suggested that the normal, healthy cumulus oophorus cells probably possessed an effective antioxidant defence system scavenging the hydrogen peroxide produced.

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The advantage of using mouse COOCs in our experiments was probably the presence of a great amount of extracellular matrix that prevented H_2O_2 from being washed out from the tissue samples during the relevant technical procedures.

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SUBMIKROSKOPICKÉ ZMĚNY V PRODUKCI PEROXIDU VODÍKU V NEKROTICKÝCH A APOPTOTICKÝCH BUŇKÁCH MYŠÍHO POSTOVULAČNÍHO CUMULUS OOPHORUS

S o u h r n

Řada nových studií prokázala vztahy mezi poškozením buněk či buněčnou smrtí a reaktivními formami kyslíku. V této studii byl prokazován peroxid vodíku in situ cytochemickou metodou založenou na jeho reakci s chloridem ceritým v cumulus oophorus myši. V normálních, funkčně aktivních buňkách kumulu nebyl peroxid vodíku prokázán, byl však pravidelně zjištěn v buňkách s ultrastrukturními známkami buněčného poškození nebo buněčné smrti. Produkce peroxidu vodíku byla velmi časným projevem těchto procesů, neboť byla zaznamenána již u nepatrných ultrastrukturních alterací buněk nebo buněčných organel a pravidelně předcházela kondenzaci jaderného chromatinu.V poškozených nebo zanikajících buňkách byla zjištěna dvě primární místa produkce peroxidu vodíku: plazmatická membrána v případě buněk zanikajících nekrózou a mitochondrie v buňkách prodělávajících apoptózu. V pozdějších fázích nekrózy byl peroxid vodíku demonstrován též na zevních mitochondriálních membránách, v pozdějších fázích apoptózy rovněž na buněčných površích. V konečných fázích apoptózy byla produkce peroxidu vodíku navíc zjištěna i na četných místech v cytoplazmě a v jádře, nebo v jaderných fragmentech a apoptotických tělískách. Nálezy dvojic buněk, z nichž jedna byla nekrotická a druhá apoptotická, a proud peroxidu vodíku mezi nimi nasvědčují tomu, že buňkami produkovaný a uvolňovaný peroxid může působit, podobně jako exogenní H_2O_2, jako chemický signál indukující smrt sousedních buněk.

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