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MORPHOLOGICAL FEATURES OF CARDIOMYOCYTE APOPTOSIS IN RATS INDUCED BY PERINATAL NITRITE HYPOXIA

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ABSTRACT

The study of apoptosis, internal mechanisms of its regulation in myocardial tissue in acute and chronic hypoxia are rather topical. Intrauterine hypoxia induces reversible and in severe cases irreversible changes in cardiomyocytes, in their sarcolemma, mitochondria and myofibrils, and in vessels too. Currently, apoptotic mechanisms attract attention as the pathway of myocardial injury. The aim of the study was to investigate the morphological features of the development of apoptotic processes in the rat myocardium at different terms of nitrite hypoxia.

The experiment was conducted on 15 white female three-month old Wistar rats weighing 180-200 g, as well as their 19 infant rats in accordance with the "Rules of the work using experimental animals". For the induction of pregnancy mature outbred male rats were set to each female at the rate of two males to four females. Experimental females were daily intraperitoneally injected sodium nitrite at a dose of 5 mg/100 g body weight (dose causing moderate hypoxia) throughout pregnancy and for 21 days (end of weaning period). During the first days (6 infant rats) and after the infant rats, which were fed with abovementioned females, reached the age of 7 days (7 infant rats) and 21 days (6 infant rats), the heart was removed under ether anesthesia.

It was found that apoptosis has the main morphological feature in the myocardial tissue of newborn rats – pronounced condensation of nuclear chromatin, which is combined with lytic lesions of mitochondria. Moreover, expansion of Ca^{2+} channels, violation of the permeability of sarcolemma developing due to the damage of cardiomyocyte membrane during hypoxia prove the penetration of lanthanum into the cell, which leads to massive cellular edema and mitochondrial damage by calcium ions Ca^{2+}. Mitochondrial membrane damage, in its turn, promotes apoptotic program launch.

Thus, the activation of cardiomyocyte apoptosis under the influence of hypoxia is the most characteristical for the neonatal period, which may promote the early elimination of defective cells. Cardiomyocyte apoptosis is rare in chronic hypoxia in the later stages of ontogeny, which is probably associated with the development of compensatory processes of adaptation.

KEYWORDS: *hypoxia, apoptosis, cardiomyocytes, mitochondria, newborns.*

INTRODUCTION

Causes of many chronic, disabling or fatal pathological conditions in adults, including the diseases of cardiovascular system, originate in the peri- and neonatal period, and in their turn, some diseases of neonatal, infant and older age are prolonged abnormalities of embryo and fetus [*Kapelko V, 2005*]. Early diagnosis and timely correction of identified pathological changes in the heart of children in their first year of life appears to be the most important factor in reducing the frequency and severity of cardiovascular disease in older children and adults. In recent years, great interest is paid towards the state of the cardiovascular system in neonates.

Currently, the mechanisms of apoptosis as one of the links in the depth and severity of myocardial damage attract cardiologists and morphologists [*Manskikh V, 2004; Kapelko V, 2005; Tsyplenkova V, Vorobyov A, 2007*]. It is proved that programmed cell death plays an important role in the formation of cardiomyopathies and in the development of arrhythmias of various origins [*Zalesski V et al., 2003; Shirokova A, 2007;*

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Zadnipryany I et al., 2014]. In the myocardium of newborns in terms of intrauterine hypoxia, reversible and in severe cases irreversible changes develop both in cardiomyocytes affecting the sarcolemma, myofibrils and mitochondria, and vessels of hemomicrocirculatory bed [*Zadnipryany I, Sataieva T, 2013*].

Despite the rapid growth in the number of publications on the role of apoptosis in the development of heart diseases there still remains unclear significance of programmed cell death and its regulation mechanisms at different terms of the myocardium development [*Shirokova A, 2007*]. There are a series of studies that suggest that hypoxia is one of the main balance "offenders" between survival and apoptosis of cardiomyocytes [*Regula K et al., 2005*]. Wherein, both the formation of high-energy phosphates in cardiomyocytes and their energy usage are disturbed. One of the mechanisms of cardiomyocytes damage during hypoxia is the violation of their membrane structures due to lipid peroxidation by abundant free radicals and peroxides [*Jäättelä M, 2002; Zadnipryany I et al., 2014*]. In this case the function of specific membrane pumps such as Na^+, K^+-ATPase, Ca^{2+}-ATPase becomes primary violated gradually increasing the permeability of the membrane up to the appearance of severe defects in it. Reported permeability latter leads to a change in the rate and direction of flow of the ions Na^+, K^+, Cl^- and H_2O, causing swelling of the cells, as well as a significant entry of calcium ions (Ca^{2+}) that leads to the discovery of so-called mitochondrial "non-specific pore", their death, and may contribute to the launch of the mitochondrial apoptosis pathway [*Manskikh V, 2004; Garg S et al., 2005*]. In this regard, the study of apoptosis, internal mechanisms of its regulation in myocardial tissue in acute and chronic hypoxia are very topical.

The aim of the study was to investigate the morphological features of the development of apoptotic processes in the rat myocardium at different terms of nitrite hypoxia.

Material and methods

Experiment is performed on 15 three-month old female white Wistar rats weighing 180-200 *g* and of 19 infant rats. The study was approved by the Bioethics Committee of the Crimea State Medical University named after S.I. Georgievsky and complies with the principles of the Guide to the Care and Use of Laboratory Animals published by the US NIH (No 85-23, revised 1985) [*International recommendations for biomedical research using animals, 1993*]. For the induction of pregnancy mature outbred male rats were set to each female at the rate of two males to four females. Experimental females were daily intraperitoneally injected sodium nitrite at a dose of 5 *mg*/100 *g* body weight (dose causing moderate hypoxia) throughout pregnancy and for 21 days (end of weaning period) [*Cherkesova D, Omarov M, 1995*]. During the first days (6 infant rats) and after infant rats, which were fed with abovementioned females, reached the age of 7 days (7 infant rats) and 21 days (infant 6 rats), after thoraco- and pericardiotomy under ether anesthesia, the heart was removed and immediately placed in a cardioplegic solution (0.9% KCl at a temperature of 0°C), which achieved heart failure in diastole. Incision of the heart for taking material from the left ventricle was performed taking into account the location of the main ways of conducting system. Preparation of material for ultramicroscopic study was carried out on the transmission electronic microscope by standard methods [*Gayer G, 1974*]. Ultrathin sections were prepared on ultratome UMTP-4 (Ukraine), stained with toluidine blue, and contrasted with uranyl acetate and lead citrate. To determine the permeability of the cell membrane, the staining technique by lanthanum hydroxide by Overton was used [*Gayer G, 1974*].

Results

Irreversible changes of cardiomyocytes were identified in newborn rats exposed to perinatal hypoxia during the first days after birth in the ultrastructural level in addition to reactive changes. Fragmentation of nuclear envelope and the increase of electron density in karyoplasm were recorded along with the intracellular edema, manifested in the form of deep invaginations of karyolemma up to the formation of segments. In this case, the nucleus took pyknotic appearance, which is regarded to be the main morphological feature of apoptosis [*Saraste A, Pulkki K, 2007*]. In apoptotic cardiomyocytes there were impaired membrane integrity of almost all the mitochondria, which were accompanied by the damage of the cristae by lytic type. There was a more pronounced

damage of sarcoplasm structures, which was followed by the formation of extensive zones of intracellular edema and vacuolization. And, as it turned out during the experiment, the fact that apoptosis affects groups consisted of 4-5 adjacent cardiomyocytes is characteristical for the early neonatal period (Fig. 1). At the same time there was a separation of damaged cells from intact ones by extending the intercalated disks. A large number of cardiomyocytes were characterized by low differentiation and small quantity of myofibrils.

Capillaries with enhanced pericapillary spaces caused by swelling phenomena were revealed. In the endothelial cells, despite the reduction in the number of secretory vesicles, there was an increase in the size of the cisterns of granular endoplasmic reticulum along with mitochondria cristae lysis resulting in reduced metabolism which cannot provide the proper level of regeneration of affected cardiomyocytes. In the lumen of capillaries a large number of lymphocytes and macrophages, which were contacting with the processes of endothelial cells, was noted.

During the ultrastructural analysis of cardiomyocytes in experimental animals, the accumulation of lanthanum oxide (La_2O_3) granules was detected in cytoplasm, as well as in the membranes and mitochondrial matrix (Fig. 2), which is considered as a marker of irreversible damage of sarcolemma, and then mitochondrial membranes.

Morphological features of apoptosis were still determined on the 7th day of life in the rat myocardium. Whereby, apoptosis either affected small groups of cardiomyocytes (2-3) or had a mosaic character. Typical feature of apoptotic damage of cardiomyocytes in these terms is a combination of destruction of almost all mitochondria with initial signs of apoptosis of cardiomyocyte nucleus (chromatin condensation, enlightenment of karyoplasm, nuclear fragmentation) (Fig. 3).

In these terms of the study, sarcoplasmic vacuolization was determined, where glycogen granules were almost absent. Marked degradation of myofibrils and lanthanum accumulation of granules were observed in the Z-damaged discs. In the sarcoplasm of damaged cardiomyocytes multiple rigor complexes were revealed. Many damaged cardiomyocytes had a large number of heterogeneous mitochondria, dilated cisterns of sarcoplasmic reticulum,

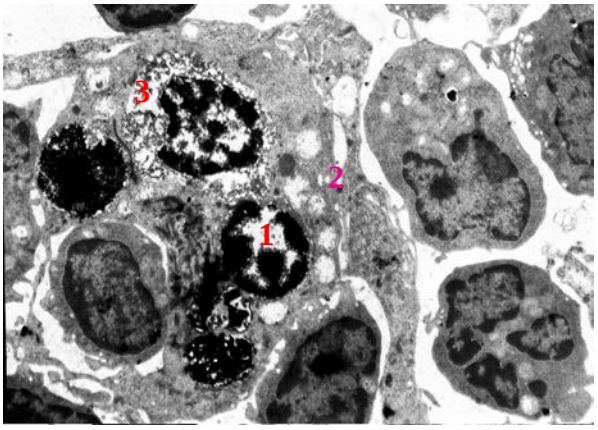

FIGURE 1. *Cardiomyocyte ultrastructure of newborn rat. Perinuclear location of nuclear chromatin and fragmentation of the cell nucleus (1), lysis of mitochondria (2), vacuolization of the cytoplasm (3). Transmission electronic microscopy×16000*

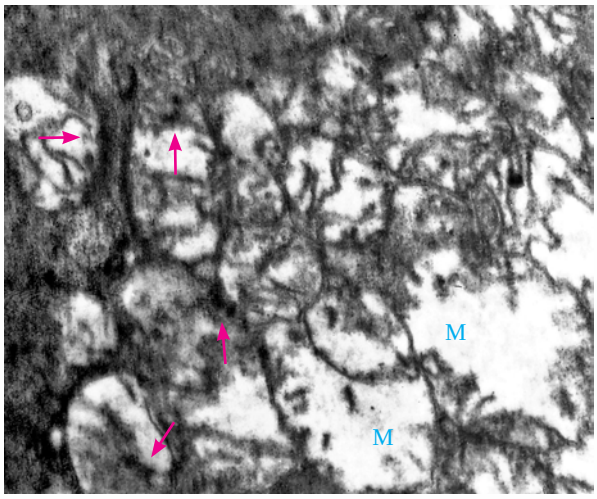

FIGURE 2. *Cardiomyocyte ultrastructure of newborn rat (0 days). Lysis of mitochondria (M), accumulation of La_2O_3 granules (shown by arrows). Transmission electronic microscopy×25000*

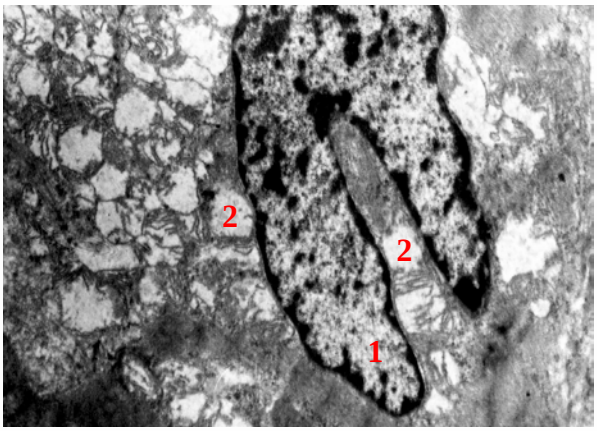

FIGURE 3. *Cardiomyocyte ultrastructure of infant rat on the 7th day. Fragmentation of the cell nucleus and chromatin condensation (1), destruction of the mitochondria cristae (2). Transmission electronic microscopy×20000*

Golgi apparatus disappearance, suggesting the depletion of compensatory-adaptive reactions in these cells. Intact cardiomyocytes still were characterized by a small number of myofibrils, i.e. low level of development of specialized organelles that was a manifestation of the specific adaptive mechanism under the threat of energy shortages.

Initial phenomenon of apoptosis was registered in some endothelial cells in the form of ring condensation of chromatin on the nuclear membrane and enlightenment of karyoplasm. There was a decrease in the number of pinocytosis vesicles of open, closed and mixed type, indicating inhibition of transport processes and, consequently, the deterioration of nutrient delivery into the interstitium. At the same time a significant increase of transport activity was noted on the surface of some endothelial cells, indicating the compensatory processes (Fig. 4).

Phenomenon of apoptosis was observed only in single cells comparison with the previous terms by the 21st day life of animals. At this stage, damaged cardiomyocytes were characterized by severe swelling of the cytoplasm by hydropic degeneration type. Their nuclear membrane formed a majority of cardiomyocytes expressed pointed intussusceptum, perinuclear space was unevenly expanded. Bundles of myofibrils in some areas were lysed. In addition, there was a swelling of heterogeneous mitochondria, which together with the large size of the matrix had a very low electron density and were located almost adjacent to karyolemma (Fig. 5). Small mitochondria in these terms were practically never found. The phenomena of hypertrophy were observed in intact cardiomyocytes that were manifested in the form of increasing the sizes of cardiomyocytes and thickening of the myofibrils, which corresponds to the long-term adaptation to the increased load.

According to the ultrastructural analysis, these periods of hypoxia are characterized by the activation of hyperplastic processes that are manifested in the form of enhanced synthesis of collagen by fibroblasts in interstitial and perivascular space, which may lead to the myocardial remodeling in the future (Fig. 6).

Thus, acute focal damage of cardiomyocytes with further organization and appearance of foci of fibrogenesis are dominating in infant rats on the 21st day of life in the use of nitrites.

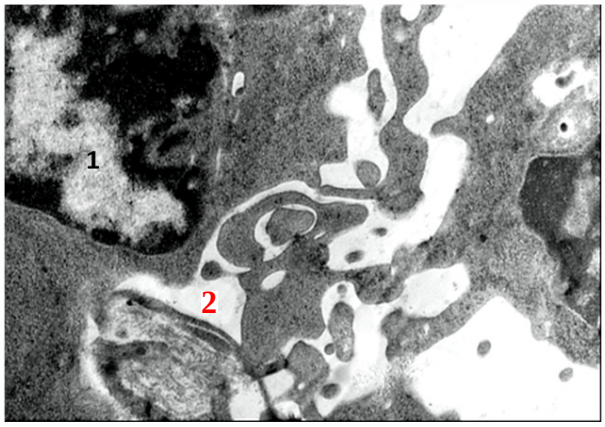

Figure 4. *Endotheliocyte ultrastructure of infant rat on the 7th day. Condensation of chromatin (1) pinocytic vesicles with amorphous content (2). Transmission electronic microscopy×15000*

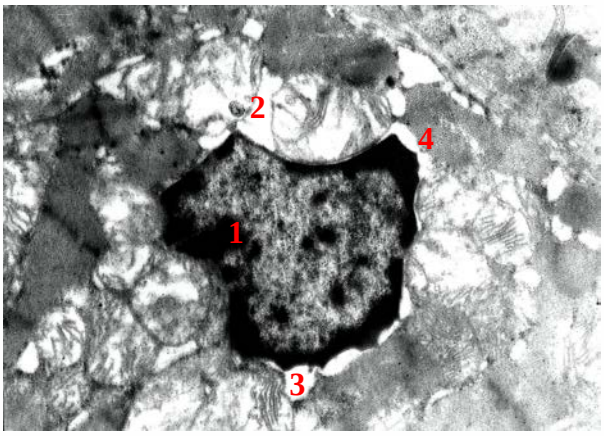

Figure 5. *Cardiomyocyte ultrastructure of infant rat on the 21st day. Fragmentation of the cell nucleus and chromatin condensation (1), destruction of the cristae of mitochondria (2), expansion of the perinuclear space (3), lysis of myofibrils, pointed invaginations of karyolemma (4). Transmission electronic microscopy×20000*

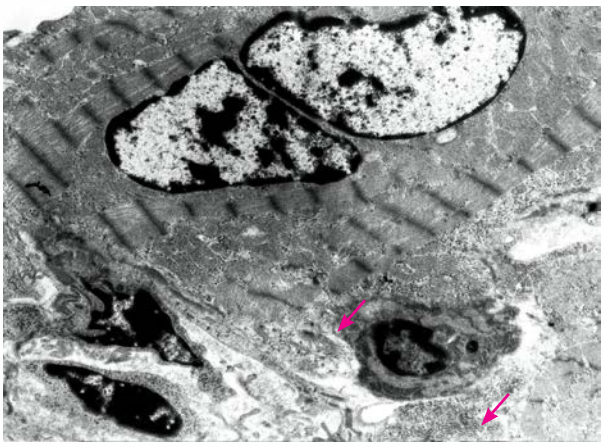

Fig. 6. *Cardiomyocyte ultrastructure of infant rat on the 21st day. Collagen fibers in the interstitium (shown by arrows). Transmission electronic microscopy×15000*

Discussion

According to the morphological data, apoptotic changes in the nucleus were observed in cardiomyocytes and, more rarely, in the endothelial cells with fully damaged mitochondria. The ultrastructural analysis of cardiomyocytes in experimental animals showed increased accumulation of La_2O_3 cytoplasmic granules and membranes in the mitochondrial matrix, which was considered as the main indicator of penetration into the sarcolemma and then mitochondrial membranes, and that, according to the literature data, is a sign of irreversible cell damage and may be one of the apoptosis triggers [*Shirokova A, 2007; Zadnipryany I, Sataieva T, 2013*].

Since all cardiomyocytes with signs of apoptosis had completely destroyed chondriome, it can be assumed, that the leading role in the development and progression of apoptosis of cardiomyocytes during hypoxia belongs to so-called mitoptosis that runs when the complete mitochondrial respiratory chain is blocked [*Zalesski V et al., 2003*]. The development of emerged intracellular acidosis contributes to the secondary deterioration of myocardial contractile function due to displacement of calcium ions from actomyosin complexes, oppression of key enzymes of glycolysis and ultrastructural changes including nuclear chromatin compaction and formation of amorphous seals in the mitochondrial matrix [*Jäättelä M, 2002*].

It is known that apoptosis inducing factor, activating endonuclease and capable to provide the chromatin condensation and fragmentation of nucleic acids, releases from mitochondria under the apoptotic signal [*Garg S et al., 2005; Kapelko V, 2005*]. If the concentration of cytochrome C and other mitochondrial proapoptotic proteins in the cytosol increases, the cell apoptosis begins that contains many defective mitochondria [*Saraste A, Pulkki K, 2007*]. Since the damaged mitochondria also produce a lot of reactive oxygen species, as a defense mechanism, the cells with damaged mitochondria are released from the tissue, which is observed during apoptosis of adjacent cardiomyocytes. These changes cause activation and subsequent inhibition of glycolysis, and respectively, reduction of adenosine triphosphate in the myocardium, which leads to disruption of its contractility [*Kapelko V, 2005*]. Whereby, metabolic end products are accumulated: lactate, hydrogen protons, inorganic phosphate and purine bases, which create considerable osmotic load in cell manifesting in the form of pronounced edema of cardiomyocytes and lysis of myofibrils. These data are consistent with the opinion of some authors, according to which the apoptosis of cardiomyocytes is the leading mechanism of arrhythmogenic right ventricular dysplasia development [*Regula K, Kirshenbaum L, 2005; Tsyplenkova V, Vorobyov A, 2007*].

Based on the foregoing, it can be assumed that profound changes of sarcolemma and mitochondria are one of the triggers of apoptotic cell death during the perinatal hypoxia. With the completion of neonatal period cardiomyocyte apoptosis occurs in rarely apparently in rats (1-5 days of life) under a nitrite hypoxia condition due to the decrease in the number of cardiomyocytes, their low differentiation and age characteristics of myocardial remodeling. In the future, it may be a theoretical basis for early corrective actions on programmed cell death of cardiomyocytes.

CONCLUSION

Apoptosis in the contractile cardiomyocytes can be provoked by the perinatal nitrite hypoxia. In the myocardial tissue from newborn rats apoptosis morphologically manifested as severe condensation of nuclear chromatin, which is combined with the lytic lesions of mitochondria. Expansion of Ca^{2+} channels, violation of the permeability of sarcolemma develops due to the damage of cardiomyocyte membranes, which is proven by the penetration of lanthanum into the cell, resulting in a marked cellular swelling and damage of mitochondrial calcium ions Ca^{2+}. Damage of mitochondrial membranes, which is also confirmed by the penetration of lanthanum in the matrix, in turn, promotes the apoptotic program launch. Activation of cardiomyocyte apoptosis under the influence of hypoxia is the most characteristical for the neonatal period, which may promote the early elimination of defective cells. Cardiomyocyte apoptosis is rare in chronic hypoxia in the later stages of ontogeny, which is probably associated with the development of compensatory processes of adaptation.

REFERENCES

1. *Cherkesova DU, Omarov MS.* [The experimental model of intrauterine nitrite hypoxia] [Published in Russian]. Bulletin. "Meditsina. Nauka i praktika". 1995; 1: 44-46.
2. *Garg S, Narula J, Chandrashekhar Y.* Apoptosis and heart failure: clinical relevance and therapeutic target. J Mol Cell Cardiol. 2005; 38(1): 73-79.
3. *Gayer G.* [Electronic histochemistry] [Published in Russian]. Moscow: Mir. 1974. 217p.
4. *International recommendations for biomedical research using animals*. Basic principles. Lanimalogiya. 1993; 1: 29.
5. *Jäättelä M.* Programmed cell death: many ways for cells to die decently. Ann Med. 2002; 34(6): 480-488.
6. *Kapelko VI.* [Evolution of concepts and metabolic basis of ischemic myocardial dysfunction] [Published in Russian]. Kardiologiia. 2005; 9: 55-61.
7. *Manskikh VN.* [Morphological methods of verification and quantification of apoptosis] [Published in Russian]. Bulletin of Siberian Medicine. 2004; 1: 63-70.
8. *Regula KM, Kirshenbaum LA.* Apoptosis of ventricular myocytes: a means to an end. J Mol Cell Cardiol. 2005; 38(1): 3-13.
9. *Saraste A, Pulkki K.* Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res. 2007; 45(3):528-537.
10. *Shirokova AV.* [Apoptosis. Signaling pathways and changes in ion and water balance of the cell] [Published in Russian]. Tsitologiya. 2007; 49(5): 385-394.
11. *Tsyplenkova VG, Vorobyov AA.* [Ultrastructural and immunohistochemical characterization of the mechanism of death of cardiomyocytes with arrhythmogenic right ventricular dysplasia] [Published in Russian]. Arkhiv patologii. 2007; 69(6): 3-7.
12. *Zadnipryany IV, Sataieva TP.* [Prospective antihypoxants in perinatal hypoxia] [Published in Russian]. Aktualnye problemy meditsiny: materialy nauch.- prakt. konf., posvyasch. 55-letiyu uchrezhdeniya obrazovaniya "Grodn. gos. med. un-t" (Actual problems of medicine: scientific-practical materials of the conference dedicated to 55th anniversary of the establishment of Grodno State Medical University). Grodno: GrSMU. 2013. pp. 210-215.
13. *Zadnipryany IV, Tretyakova OS, Sataieva TP.* [Perinatal hypoxia as an inducer of apoptosis in neonatal cardiomyocytes] [Published in Russian]. World of Medicine and Biology. 2014; 43(1):169-176.
14. *Zalesski VN, Stadnyuk LA, Velikaya NV.* [Apoptotic and autophagic cell death in the way of hypertrophy and myocardial remodeling] [Published in Russian]. Journal of Medical Sciences of Ukraine. 2003; 9(4):699-712.