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Tissue immunostaining of growth, pro- and anti-apoptotic biomarkers in myocardial samples from newborns with hypoxic injury

Imunoexpressão tecidual de biomarcadores de crescimento, pró e antiapoptóticos, em amostras de miocárdio de recém-nascidos com lesão hipóxica

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ABSTRACT

Introduction: Despite the importance of cardiovascular diseases to population health, there is limited knowledge about how neonatal hypoxia, related or not with prematurity, may cause cell injury to cardiomyocytes in the neonatal period and what the consequences to adult life are. **Objective:** To analyze the tissue immunostaining of biomarkers involved in the process of cell death and growth in the myocardium of hypoxic newborns. **Method:** Human myocardium samples (left ventricle) from necropsies of hypoxic newborns were organized in multi-sample blocks and submitted to immunohistochemical reactions by the immunoperoxidase technique. The primary antibodies used were anti-B-cell lymphoma 2 (Bcl2)-associated X protein (BAX), anti-mitofusin-2 (Mfn2), anti-tumor necrosis factor receptor-associated protein 1 (TRAP1), anti-Bcl2, anti-angiotensin II, anti-serine/threonine (Akt) 1, anti-Akt2 and anti-Akt3. Tissue immunostaining data were correlated with clinical data (gender, weight, gestational age, first-minute and fifth-minute Apgar score, arterial blood pH and survival time) and pathological data (death cause and primary disease). **Results:** The average tissue immunostaining of BAX was 25.61%; TRAP1 was 7.86%, angiotensin II was 1.24%, Akt2 was 16.35% and Akt3 was 20.61%. The biomarkers Akt1, Mfn2 and Bcl2 presented very low or absent tissue immunostaining in most cases of this study. There was no correlation between the average biomarker tissue immunostaining and the survival time or any other clinical or pathological factor studied. **Conclusion:** These data seem to strengthen the coordinated action of the pro-apoptotic, anti-apoptotic and cell growth biomarkers in the myocardium of hypoxic newborns in order to determine the degree of lesion or cell death and the tissue recovery capacity.

Key words: newborn; immunohistochemistry; cell enlargement; apoptosis; cell proliferation.

INTRODUCTION

Increased risk of morbidity and mortality of newborns is associated with maternal, fetal and neonatal conditions, and one of the most important factors is prematurity[1]. Prematurity can lead the newborn to countless problems due to the difficulty of extrauterine adaptation caused by the immaturity of their organs and systems, such as cardiovascular and respiratory problems that are important in the process of hypoxia and death[1, 2].

Although it is common knowledge that cardiovascular diseases play an important role in population health and life expectancy, there is limited knowledge of how adverse conditions experienced by newborns, such as perinatal hypoxia, can cause injury to cardiomyocytes and how this can interfere in their adult life.

In order to understand the phenomena related to perinatal hypoxia and death of cardiomyocytes, the aim of this study is to evaluate, in myocardial samples from newborns, the tissue immunostaining of growth and cell death biomarkers, and to correlate them with clinical and pathological variables[3, 4].

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METHOD

In this study, necropsy samples of human myocardium (left ventricle) were used, taken from the Pediatric Necropsy Sector at the Pathology Service of Hospital das Clínicas of Universidade Federal do Paraná (HC-UFPR), between the years of 1991 and 2007. This project was approved by the Ethics Research Committee of UFPR under the number 2533.140/2011-06.

The cases included were necropsies of viable (gestational age of 24 weeks or more) newborns (0-28 days of life), with perinatal hypoxia, premature or not, of both genders. The pathological and clinical criteria of perinatal hypoxia were defined as: fifth-minute Apgar < 6 and/or pH < 7.2 and/or necropsy exam with signs of perinatal hypoxia, such as serous and mucosa petechiae, polivisceral systemic congestion, diffuse alveolar damage and germinal matrix hemorrhages. Inadequate samples or records considered incomplete were excluded from this study.

The clinical variables collected at the records were gender, weight (in grams), gestational age (in weeks), first- and fifth-minute Apgar, arterial blood pH, and survival (in hours or days of life). The pathological variables collected in the necropsy reports were cause of death and primary disease.

Paraffin blocks of 154 samples of left ventricle myocardium were used and prepared in 13 tissue microarray (TMA) blocks, with about 12 cases each. The cases were represented by two samples, with 3 mm diameter each sample. The total area analyzed was 14 mm^2 for each case ($A = \pi.r^2 \times 2$ samples). Then, the TMA blocks were cut in multi-sample slides to perform the immunohistochemical assays.

The technique used for immunohistochemistry reactions was the immunoperoxidase described by Carmo Debur (2010) *et al.*[5]. The positive and negative controls were stained in all reactions of this study.

The following biomarkers were selected: B-cell lymphoma 2 (Bcl2)-associated X protein (BAX) and mitofusin-2 (Mfn2), which are pro-apoptotic proteins; tumor necrosis factor receptor-associated protein 1 (TRAP1) and Bcl2, which are anti-apoptotic proteins; and angiotensin II and serine/threonine (Akt) 1, 2 and 3, which are involved with cell growth.

The primary antibodies used were: anti-BAX, rabbit polyclonal, dilution 1:50, and anti-Bcl2, mouse monoclonal, dilution 1:200, both from Dako® (Glostrup, Denmark); anti-Mfn2, rabbit monoclonal, dilution 1:100, anti-TRAP1, rabbit monoclonal, dilution 1:400, anti-angiotensin II, rabbit polyclonal, dilution 1:400, anti-Akt1, rabbit polyclonal, dilution 1:200, rabbit polyclonal, dilution 1:200, and anti-Akt3, rabbit polyclonal, dilution 1:400, all from Abcam® (Cambridge, USA). Positive (left ventricle myocardium samples from adults) and negative controls (by primary antibody omission) were immunostained for each reaction.

The slides were read using the optical microscope Olympus® BX50 (Tokyo, Japan), coupled to a video camera Dino-Eye and to a computer with image analysis software Image Pro Plus™ (Maryland, USA). Eight images were captured in high-power field [(HPF) = 400×] for each case and for each studied biomarker. The area of each image was 115,226.1 μm^2, with resolution of $1,024 \times 768$ pixels.

The positive control of each reaction was scanned, and an image in HPF was chosen as "mask", containing the appropriate positivity for each chosen biomarker. The mask was then superimposed to the digital images of all cases. Based on the ideal immunopositivity of the mask, the analysis software Image Pro Plus™ calculated the immunopositive area of each image and transformed these data into immunopositive area per square micrometer (μm^2). This area in μm^2 was then divided by the constant 115,226.1 μm^2, which was the total area of the field evaluated, generating a percentage of immunopositive area by HPF. An average area percentage in eight HPFs was calculated for each case.

The statistical analysis evaluated the association between two qualitative variables, considering Fisher's exact test or chi-square test. The comparison between two groups in relation to quantitative variables was drawn using Student's *t* test for independent samples or the non-parametric test of Mann-Whitney. Values of $p < 0.05$ were considered statistically significant. Data were analyzed with the computer program IBM SPSS Statistics v.20.

RESULTS

There was a discreet predominance of male gender, with 56.5% of the cases ($n = 87$); premature infants were majority, with 78.6% ($n = 121$).

The necropsy hypoxia signs in the pathological exam were reported as cause of death in 86.4% ($n = 133$), whereas the other 13.6% of the sample ($n = 21$) presented clinical criteria of perinatal hypoxia. The hyaline membrane disease was the most common primary disease in this study, with 47.1% of the cases ($n = 72$).

The clinical parameters weight, gestational age, first- and fifth-minute Apgar, arterial blood pH and survival time, as well

as average percentage of immunostaining of BAX, TRAP1, angiotensin II, Akt2 and Akt3 by HPF, are shown in **Table 1**.

The Bcl2 presented tissue immunopositivity in just one case, the Mfn2 presented weak immunopositivity in only 24 cases, and the Akt1 did not present tissue immunostaining in any case of this study.

The association between survival time and the biomarkers was not significant (**Table 2**).

When correlating the median of the tissue immunostaining of BAX, angiotensin II, Akt2, Akt3 and TRAP1 with weight, gestational age, first- and fifth-minute Apgar, pH and death cause, there was no significant statistical values.

TABLE 1 – Pathological and clinical parameters of the sample and tissue immunostaining of the biomarkers in percentage of immunopositive area by HPF

Variable	*n*	Median	Average	SD
Weight (grams)	139	1,480	1647	911.7
Gestational age (weeks)	154	33	32.5	4.6
Apgar 1	144	2.5	3.5	2.9
Apgar 5	144	5	5.3	3.1
pH	93	7.1	7	0.2
Survival (days)	154	1	4.2	6
BAX	149	24.57%	25.61%	8.53%
TRAP1	141	3.54%	7.86%	8.33%
Angiotensin 2	151	0.89%	1.24%	1.21%
Akt2	147	16.12%	16.35%	10.57%
Akt3	151	19.94%	20.61%	7.58%

HPF: high-power field; SD: standard deviation; BAX: B-cell lymphoma 2 (Bcl2)-associated X protein; TRAP1: tumor necrosis factor receptor-associated protein 1; Akt: serine/threonine.

TABLE 2 – Relationship between survival time (days or hours) and tissue immunostaining of the biomarkers in percentage of immunopositive area by HPF

Marker	Survival	*n*	Average (%)	Median (%)	SD (%)	*p* value*
BAX	≤ 24 hours	87	24.76	22.04	8.7	0.263
	> 1 and ≤ 7 days	32	25.96	25.74	7.39	
	> 7 days	30	27.68	26.51	9.05	
TRAP1	≤ 24 hours	85	7.64	2.9	8.07	0.584
	> 1 and ≤ 7 days	30	7.23	0.83	8.57	
	> 7 days	26	9.3	8.59	9.05	
Angiotensin	≤ 24 hours	88	1.08	0.97	0.83	0.152
	> 1 and ≤ 7 days	33	1.49	1	1.57	
	> 7 days	30	1.43	0.76	1.62	
Akt2	≤ 24 hours	85	15.3	15.48	10.14	0.222
	> 1 and ≤ 7 days	33	16.32	13.61	11.49	
	> 7 days	29	19.3	18.42	10.56	
Akt3	≤ 24 hours	88	20.59	20.08	10.56	0.940
	> 1 and ≤ 7 days	33	20.95	21.59	8.72	
	> 7 days	30	20.27	18.91	8.42	

**: Anova with one factor, p < 0.05; or non-parametric Kruskal-Wallis test, p < 0.05.*

SD: standard deviation; BAX: B-cell lymphoma 2 (Bcl2)-associated X protein; TRAP1: tumor necrosis factor receptor-associated protein 1; Akt: serine/threonine.

Significant statistical correlations were not found when the biomarkers were compared two by two.

The immunohistochemical aspects of this study are represented in the **Figure**. The anti-Akt1, anti-Bcl2 and anti-Mfn antibodies presented very weak or even negative reaction in the majority of the cases in this study. Just 24 cases were weakly positive with Mfn2, and all the other cases were totally negative. Regarding Bcl2, just one case was weakly positive, and the other cases were totally negative. The anti-Akt1 antibody was totally negative in all cases. The anti-Akt2 and anti-Akt3 antibodies presented moderate cytoplasmic and nuclear immunopositivity in the majority of the cardiomyocytes in all cases. The anti-BAX antibody presented moderate cytoplasmic immunopositivity in the majority of the cases.

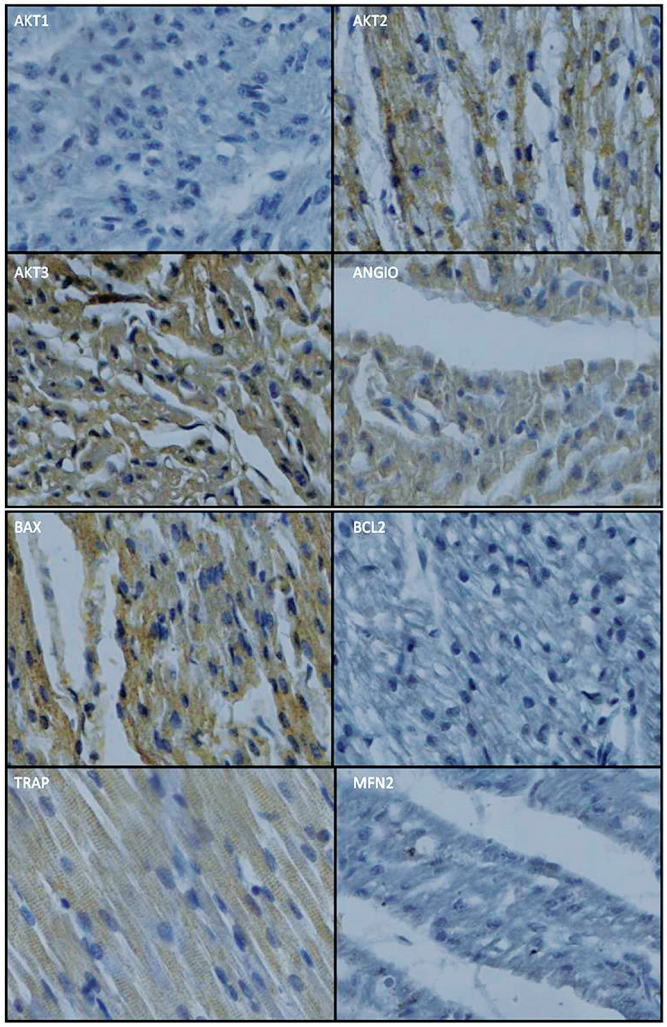

FIGURE – *Histological sections immunostained with the antibodies anti-BAX, Bcl2, TRAP1, Mfn2, Akt1, Akt2, Akt3 and angiotensin II*

BAX: B-cell lymphoma 2 (Bcl2)-associated X protein; TRAP1: tumor necrosis factor receptor-associated protein 1; Mfn2: mitofusin-2; Akt: serine/threonine.

DISCUSSION

At birth, the lungs of the newborn substitute the placenta as the organ of gas exchange, and the central respiratory activity becomes finely adjusted to the metabolic necessities of the organism. Some respiratory disorders occur due to a failure of normal cardiopulmonary adaptation to the new environment[1].

Many circumstances are involved in the appearance of hypoxia, such as apnea at birth, transient tachypnea of the newborn, hyaline membrane disease and persistent fetal circulation, and other congenital or acquired diseases. Any of these situations may generate a hypoxemic state that may lead to serious consequences to the newborn, with increased life risk[1, 6]. In our sample, the pathological evidences of perinatal hypoxia were the main cause of death, and the main underlying disease as a cause of perinatal hypoxia was the hyaline membrane disease related to prematurity.

Xue *et al*. (2009) demonstrated the association between adverse events in the perinatal period and increased risk of ischemic heart disease in adulthood. Another study showed that the heart responds to different stress signs, including hypoxia, what causes a variety of functional responses in cardiomyocytes, such as death by apoptosis and cell growth[2, 7].

Cell death, also known as apoptosis, is an important mechanism in the development of organs and in homeostasis and remodeling of tissues. It can occur from a number of stimuli, and one of the most important is oxidative stress, closely linked to hypoxia episodes[4].

One apoptosis biomarker widely studied is the protein BAX, which is a pro-apoptotic member of the Bcl2. In healthy cells, it resides in the cytosol, in a monomeric form. However, after induction of apoptosis, it changes its conformation and it is translocated to the mitochondria, which forms a pore in the membrane, allowing leakage of cytochrome c, phase in which apoptosis becomes irreversible[4]. Capano and Crompton demonstrated that translocation of the BAX to the mitochondria is a gradual process that begins in the first 20 minutes of a hypoxic event and progresses about three hours in the cardiac cell. This accumulation of BAX, then, culminates in apoptosis[4].

It is known that the Bcl2 can prevent apoptosis by inhibiting the capacity of the BAX to release cytochrome c from the mitochondrias[8]. Protein Bcl2 is also involved in the decrease of apoptotic events, because it acts inhibiting the activation of pro-caspase by blocking the action of BAX[9]. Thus, Bcl2 plays an important role in the regulation of function and metabolism of the mitochondria. Although it is expressed at low levels in cardiomyocytes, this potent modulator of cell death is a promising therapeutic agent for heart diseases since it has the capacity to reduce cell death during ischemia[10]. Its anti-apoptotic action in cardiomyocytes is taken by blocking p53 and increasing the capacity of the mitochondria to resist to high levels of calcium. The interaction between the two proteins, BAX and Bcl2, will determine whether there will be stimulation or blocking of apoptosis[9, 10].

Our results suggest an involvement of proteins BAX and Bcl2 in the process of apoptosis caused by the hypoxia of cardiomyocytes because we observed decreased tissue expression of the anti-apoptotic protein Bcl2 and an increased pro-apoptotic protein BAX.

Another anti-proliferative and pro-apoptotic biomarker used in our study is Mfn2, also called hyperplasia suppressor, due to its anti-proliferative effects. It is also an endogenous inhibitor of the Rat sarcoma virus (*Ras*), a known oncogene related to cell proliferation pathways[11]. Studies show that the protein Mfn2 is located in the outer membrane of the mitochondria and it participates in the mitochondrial fusion, and also causes suppression of cell growth by inhibiting the signaling pathway Ras-extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK)[11]. The absence or reduction in the expression of Mfn2 promotes an increase in cell proliferation, contributing to the development of many vascular proliferative disorders. Moreover, when high rates of Mfn2 are associated with the presence of the protein BAX, promotion of early stages of apoptosis[11] is observed. It is known that the protein Mfn2 is an essential factor in regulating the survival of cardiac muscle cells by unknown mechanisms. Results indicate that the increase in its expression in cardiomyocytes is related to apoptosis induced by oxidative stress, thus it acts in hypoxic situations[11].

In our study, Mfn2 presented low tissue immunoexpression. This fact suggests that this protein does not participate in the processes of apoptosis mediated by hypoxia in newborns. Furthermore, there was no positive correlation in the expression of BAX and Mfn2 in this study. This fact may suggest that this association does not happen in the myocardium of hypoxic newborns.

Tissues also have an anti-apoptotic mechanism that prevents excessive loss of cells. In a hypoxic event, all these mechanisms work and the result of these factors determines which cells undergo apoptosis and which are able to adapt, with or without cell proliferation[12].

Mitochondrias are the first target of the hypoxic damage in cardiomyocytes. Many factors, such as calcium

extravasation, increase in reactive oxygen species and decrease in adenine contribute to mitochondrial damage during hypoxia. Mitochondrial dysfunction may directly cause cell death after a hypoxic event[12]. This happens due to mitochondrial permeability of the transition pore (PTPM), which is a non-specific pore that opens when there is calcium extravasation, oxidative stress, adenine depletion or increased phosphate levels situations. Once opened, the membrane potential and the cell pH gradient are dissipated, and these changes may lead to cell death[12].

In this study, we analyzed the tissue expression of the anti-apoptotic protein TRAP1. Studies showed that the increased expression of TRAP1, a mitochondrial chaperone, member of the family heat-shock proteins 90 (Hsp90), acts in the integrity of the mitochondrial membrane potential, maintains the level of ATP production, and preserves cell viability in ischemic damage episodes[13, 14]. The molecule TRAP1 is located in the mitochondrial matrix, with a fraction distributed in the intermembrane space. In some tissues, an extramitochondrial localization was observed, as in the insulin granules in the pancreas, sarcomeres of cardiomyocytes, pancreatic and heart cell cores and endothelium, but there is not a clear understanding of its function in this situation[15]. The first function assigned to TRAP1 is protection against apoptosis via the mitochondria. It was identified, in studies with antitumor agents, that when tumor cells were treated with these agents, there was a decrease in the expression of TRAP1, associated with an increase in apoptosis via the mitochondria[13]. The role of TRAP1 in response to hypoxia was also studied, and it was demonstrated that an increased expression of TRAP1 has a protective effect against damage caused by hypoxia in cardiomyocytes, whereas when TRAP1 is inactive, there is an increase in cell death, reduction in cell viability and reduction in the mitochondrial membrane potential. This led to the conclusion that hypoxia induces an increased expression of TRAP1 in cardiomyocytes, and TRAP1 has an important role in cellular protection and maintenance of mitochondrial function[12].

Our study showed good tissue immunostaining of TRAP1, suggesting that it could be one of the protective factors of cardiomyocytes against damage caused by hypoxia.

Studies indicate that perinatal hypoxia in cardiomyocytes can also result in cell growth by hyperplasia, hypertrophy and remodeling as an immediate response to injury, fact that seems to extend into adulthood as a late response[7]. Among the markers of hypertrophy in cardiomyocytes, we studied angiotensin II, which increases the expression of myocardin or induces cardiac hypertrophy directly[7]. The latest findings showed that hypoxia in cardiomyocytes of newborns increases the expression of myocardin, apparently mediated by the increased expression of proteins of angiotensin II pathway and ERK, and all these factors are directly related to myocardial hypertrophy, even in adulthood[7].

The results presented in this study showed good tissue immunostaining of angiotensin II, suggesting its participation in the hypertrophy process as an immediate response to injury in the hypoxic myocardium from newborns. The postnatal growth of myocardium results from the combined action of hyperplasia and hypertrophy, and hyperplasia greatly decreases in the adult heart[16].

Another important biomarker of hypertrophy and hyperplasia of cardiomyocytes studied in our research is the Akt, a serine/threonine kinase with potent anti-apoptotic actions both *in vitro* and *in vivo*. It is associated with promotion of cell proliferation (hyperplasia) in many non-cardiac cell types, including oncogenic transformation[16]. Some studies have shown that Akt is responsible for promoting cell proliferation in the postnatal heart. This occurs due to the nuclear accumulation of Akt, which expands the population of cardiomyocytes in cell division as well as the number of myocardial progenitor cells[16].

The absence of tissue immunostaining of Akt1 protein, in this study, suggests that it is not part of the stimulus process of myocardial hyperplasia in response to injury, at least in the studied population of hypoxic newborns. On the other hand, we observed high tissue expression of Akt2 and Akt3, suggesting that these two proteins could participate in the stimulation of hyperplasia in the myocardium of hypoxic newborns.

CONCLUSION

These data seem to strengthen the coordinated action of pro-apoptotic, anti-apoptotic and cell growth biomarkers in the myocardium of hypoxic newborns in order to determine the degree of lesion or cell death and tissue recovery capacity.

The biomarkers BAX, TRAP1, angiotensin II, Akt2 and Akt3 presented good tissue immunoexpression in samples of hypoxic newborn myocardium.

The biomarkers Akt1, Mfn2 and Bcl2 presented low or absent tissue immunoexpression in samples of this study.

There was no correlation between tissue immunoexpression of the biomarkers studied with clinical and pathological data of the sample.

RESUMO

Introdução: *Apesar da importância de novas descobertas em patofisiologia das doenças cardiovasculares para a saúde da população, ainda existe pouco conhecimento sobre como a hipóxia neonatal, relacionada ou não com a prematuridade, pode causar lesão celular nos cardiomiócitos no período perinatal e quais são as consequências para a vida adulta.* ***Objetivo:*** *Analisar a imunoexpressão tecidual de biomarcadores envolvidos no processo de morte e crescimento celular em miocárdio de recém-nascidos hipoxemiados.* ***Método:*** *Amostras de miocárdio humano (ventrículo esquerdo), provenientes de necrópsias de neonatos hipoxemiados, foram organizadas em blocos multiamostrais e submetidas a reações imuno-histoquímicas pela técnica da imunoperoxidase. Os anticorpos primários utilizados foram antilinfoma de células B tipo 2 (Bcl2), antiproteína X associada ao Bcl2 (BAX), antimitofusina 2 (Mfn2), antiproteína 1 relacionada com o receptor ao fator de necrose tumoral (TRAP1), antiangiotensina II, antiproteína quinase 1 (Akt1), antiproteína quinase 2 (Akt2) e antiproteína quinase 3 (Akt3). Os dados de imunoexpressão tecidual foram correlacionados com os dados clínicos (gênero, peso, idade gestacional, índice de Apgar do primeiro e quinto minuto, pH do sangue arterial e sobrevida) e anatomopatológicos (causa da morte e doença de base).* ***Resultados:*** *A média de imunoexpressão tecidual do BAX foi 25,61%; do TRAP1, 7,86%; da angiotensina II, 1,24%; do Akt2, 16,35%; e do Akt3, 20,61%. Os biomarcadores Akt1, Mfn2 e Bcl2 apresentaram imunoexpressão tecidual muito baixa ou ausente na maioria dos casos deste estudo. Não houve correlação da média de imunoexpressão tecidual dos biomarcadores com o tempo de sobrevida ou outro fator clínico ou anatomopatológico estudado.* ***Conclusão:*** *Esses dados parecem fortalecer a ação coordenada de biomarcadores pró e antiapoptóticos e de crescimento celular em miocárdio de recém-nascidos hipoxemiados a fim de determinar o grau de lesão ou morte celular e a capacidade de recuperação tecidual.*

Unitermos: *recém-nascido; imuno-histoquímica; hipertrofia; apoptose; proliferação de células.*

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