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Effect of preterm birth in rats on proliferation and hyperplasia of cardiomyocytes

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ABSTRACT

Aim. To identify the effect of preterm birth on proliferation and hyperplasia of cardiomyocytes in the early postnatal period of ontogenesis in rats.

Materials and methods. Preterm birth (on day 21 and 21.5 of gestation) in Wistar rats was induced by subcutaneous administration of mifepristone. Immunohistochemistry was used to identify and calculate the number of Ki67-positive and Mklp2-positive cardiomyocytes in the left ventricle of preterm and full-term rats on days 1, 2, 3, 4, 5, and 6 of postnatal ontogenesis. Statistical analysis of morphometric parameters was performed using the Shapiro – Wilk test and Mann – Whitney test with the Bonferroni correction.

Results. We revealed an increase in the number of Ki67-positive cardiomyocytes in the left ventricle of the rats: on day 1 of postnatal ontogenesis (in the rats born on day 21 of gestation) and on days 3–5 of postnatal ontogenesis (in the rats born on day 21.5 of gestation). Preterm birth in rats did not result in a change in the number of Mklp2-positive cardiomyocytes in the left ventricular wall.

Conclusion. A change in the pattern of Ki67 expression by cardiomyocytes in the rats born 12 or 24 hours before full term was demonstrated in the early postnatal period of ontogenesis. An isolated increase in Ki67 expression without a change in Mklp2 expression by cardiomyocytes in the left ventricular wall of preterm rats indicates acceleration of cardiomyocyte hypertrophy. Shorter duration of prenatal development is associated with more pronounced morphological and functional rearrangements in the rat myocardium.

Keywords: preterm birth; cardiomyocyte; proliferation; hyperplasia; hypertrophy, experiment

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Влияние преждевременного рождения крыс на пролиферацию и гиперплазию кардиомиоцитов

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РЕЗЮМЕ

Цель. Установить влияние преждевременного рождения на процессы пролиферации и гиперплазии кардиомиоцитов в раннем постнатальном периоде онтогенеза у крыс.

Материалы и методы. Преждевременные роды (на 21-е и 21,5-е сут беременности) крыс линии Вистар индуцировали подкожным введением мифепристона. Иммуногистохимически в левом желудочке преждевременно рожденных и доношенных крыс на 1, 2, 3, 4, 5 и 6-е сут постнатального периода онтогенеза выявляли и подсчитывали количество Ki67-позитивных и Mklp2-позитивных кардиомиоцитов. С помощью критерия Шапиро – Уилка и критерия Манна – Уитни с поправкой Бонферрони провели статистический анализ морфометрических показателей.

Результаты. Продемонстрировано увеличение количества Ki67-позитивных кардиомиоцитов в левом желудочке сердца крыс: на 1-е сут постнатального периода онтогенеза (у рожденных на 21-е сут беременности) и на 3–5-е сут постнатального периода онтогенеза (у рожденных на 21,5-е сут беременности). Преждевременное рождение не приводит к изменению количества Mklp2-позитивных кардиомиоцитов в стенке левого желудочка крыс.

Заключение. В раннем постнатальном периоде онтогенеза продемонстрировано изменение паттерна экспрессии Ki67 кардиомиоцитами крыс, рожденных на 12 или 24 ч ранее срока. Изолированное увеличение экспрессии Ki67 без изменения экспрессии Mklp2 кардиомиоцитами в стенке левого желудочка преждевременно рожденных крыс свидетельствует об акселерации гипертрофии кардиомиоцитов. Меньшая продолжительность внутриутробного периода развития ассоциирована с более выраженными морфофункциональными перестройками миокарда крыс.

Ключевые слова: преждевременное рождение, кардиомиоцит, пролиферация, гиперплазия, гипертрофия, эксперимент

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Preterm birth (birth before 37 completed weeks of gestation) increases the risk of early development of cardiovascular diseases [1, 2], such as coronary heart disease [3], hypertension, and heart failure [4]. Preterm birth is associated with structural and functional immaturity of organs, which is the reason for their adaptive morphogenesis in the postnatal period. Thus, there are no differences in the heart structure between full-term and preterm children in the prenatal period; initial structural changes in the heart in preterm children emerge in the postnatal period [5]. It is known that the morphological and

functional features of the heart in preterm children are determined already at the third month of the postnatal period of ontogenesis: an increase in the relative mass of the right and left ventricles is observed, compared with that in full-term infants [5]. Over time, the structural features of the heart in preterm children are preserved [6] and serve as a prerequisite for earlier development of cardiovascular diseases in this group of individuals [7].

It remains unclear how preterm birth leads to the formation of structural and functional features of the heart, though researchers pay close attention to this problem [8]. At the same time, correction of structural features in the heart of preterm children in the early postnatal period of ontogenesis can prevent the development or reduce the risk of cardiovascular diseases in adulthood [9–11].

Determination and detailed characterization of morphological and functional changes in the heart of preterm children in the early postnatal period are priority tasks, which solution is hampered by the lack of histologic studies. Single studies devoted to the histologic characteristics of the myocardium in preterm children are difficult to interpret, since the hearts of stillborn babies are used as controls [12], while it is adaptation of the heart and blood vessels to the conditions of *ex utero* functioning that is of great interest. Therefore, it is relevant to identify and study the dynamics and consequences of postnatal morphological and functional features of the heart in preterm animals in the experiment. The aim of the study was to establish the effect of preterm birth on proliferation and hyperplasia of cardiomyocytes in the early postnatal period of ontogenesis in rats.

MATERIALS AND METHODS

Wistar rats of both sexes were used in the experiment. A full gestation period for Wistar rats is 22 days. Rats born two days before full term are not viable. Rats born one day before full term are characterized by a sufficient degree of structural and functional immaturity of organs and are used as models for studying the effects of preterm birth [13, 14]. Based on clinical data, according to which the severity of the morphological and functional changes in the heart and blood vessels correlates with the degree of prematurity [15, 16], as well as on the information that even a moderate degree of prematurity leads to impaired functioning of the cardiovascular system in adulthood [17], we formed two groups of rats in the study: rats born 12 and 24 hours before full term, respectively (Table 1).

Table 1

Characteristics of the experimental groups

Experimental group	Duration of prenatal development in experimental animals, days	Time of mifepristone injection, days of pregnancy
Control group, $n = 30$	22.0	–
Group 1, $n = 30$	21.5	20.5
Group 2, $n = 30$	21.0	20.0

The offspring were obtained from the intact male (aged 2 months, 180 ± 20 g) and female (aged 3 months, 180 ± 20 g) Wistar rats, the latter were kept in individual cages. In females, the phase of the estrous cycle was determined daily. In the proestrus, a male was placed with a female for a night. The following morning, the male was removed from the cage, and vaginal smear of the female rat was analyzed to verify coitus. The day of detection of spermatozoa in the vaginal smear was considered to be the first day of pregnancy. Pregnant females were kept in individual cages and fed with LbK 120 R-22 feed for pregnant laboratory rodents (Delta Feeds, Russia). Preterm labor was induced on days 21 and 21.5 of pregnancy by subcutaneous administration of mifepristone (1 ml, 10 mg / kg of body weight, Sigma-Aldrich, USA) to the rats [18].

The rats were euthanized on days 1, 2, 3, 4, 5, and 6 of postnatal ontogenesis by CO_2 inhalation. The rat heart was fixed in the buffered (pH 7.4) formalin solution (BioVitrum, Russia) for 24 h, then it was washed in running water, dehydrated in the Isoprep solution (BioVitrum, Russia), and embedded in the HISTOMIX paraffin mixture (BioVitrum, Russia). The sections obtained on the automatic microtome (HM355S, Thermo Fisher Scientific, China) were used for immunohistochemistry. Ki67 (a proliferation marker) and Mklp2 (a cytokinesis marker) were detected on the sections by the indirect peroxidase method. Ki67 is non-specific for determining true mitosis and is expressed during endomitosis as well [19]. On the contrary, Mklp2 (mitotic kinesin-like protein 2) is a marker of cytokinesis, the final stage of true mitosis. Therefore, Mklp2 makes it possible to identify cells undergoing the final stages of mitosis [20].

To unmask antigens, the deparaffinized sections were exposed to high temperatures in the citrate buffer (0.01 M, pH 6.0). Ab16667 (Anti-Ki67 antibody [SP6],

1:300) and bs-7750r (Anti-Mklp2 antibody, 1:500) were used as primary antibodies. Primary antibodies were visualized using the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit – Micro-polymer (Abcam, UK). After the immunohistochemical staining, the sections were counterstained with Gill's hematoxylin.

Immunohistochemical slides were studied using the Axioscope 40 light microscope (Zeiss, Germany) and the Canon PowerShot G5 digital camera (Canon, China). To determine the localization of immunopositive cardiomyocytes, the thickness of the left ventricular myocardium was arbitrarily divided into three parts: subepicardial, middle, and subendocardial. The number of Ki67-positive and Mklp2-positive cardiomyocytes per 1 mm^2 of the left ventricular wall section area was counted.

Statistical analysis was carried out using SPSS 16.0 (IBM, USA). The Shapiro –Wilk test and the Mann – Whitney test with the Bonferroni correction were used. The results of the morphometric study were presented as the median and the interquartile range Me $(Q_1; Q_3)$. The differences were statistically significant at $p < 0.01$.

RESULTS

In the left ventricular myocardium of rats, Ki67 and Mklp-2 were detected in the cytoplasm of different cells: endotheliocytes, fibroblasts, and cardiomyocytes (Figure). In the observed periods, Ki67-positive cardiomyocytes were diffuse in the left ventricular myocardium in all experimental groups, while Mklp2-positive cardiomyocytes had predominantly subendocardial localization.

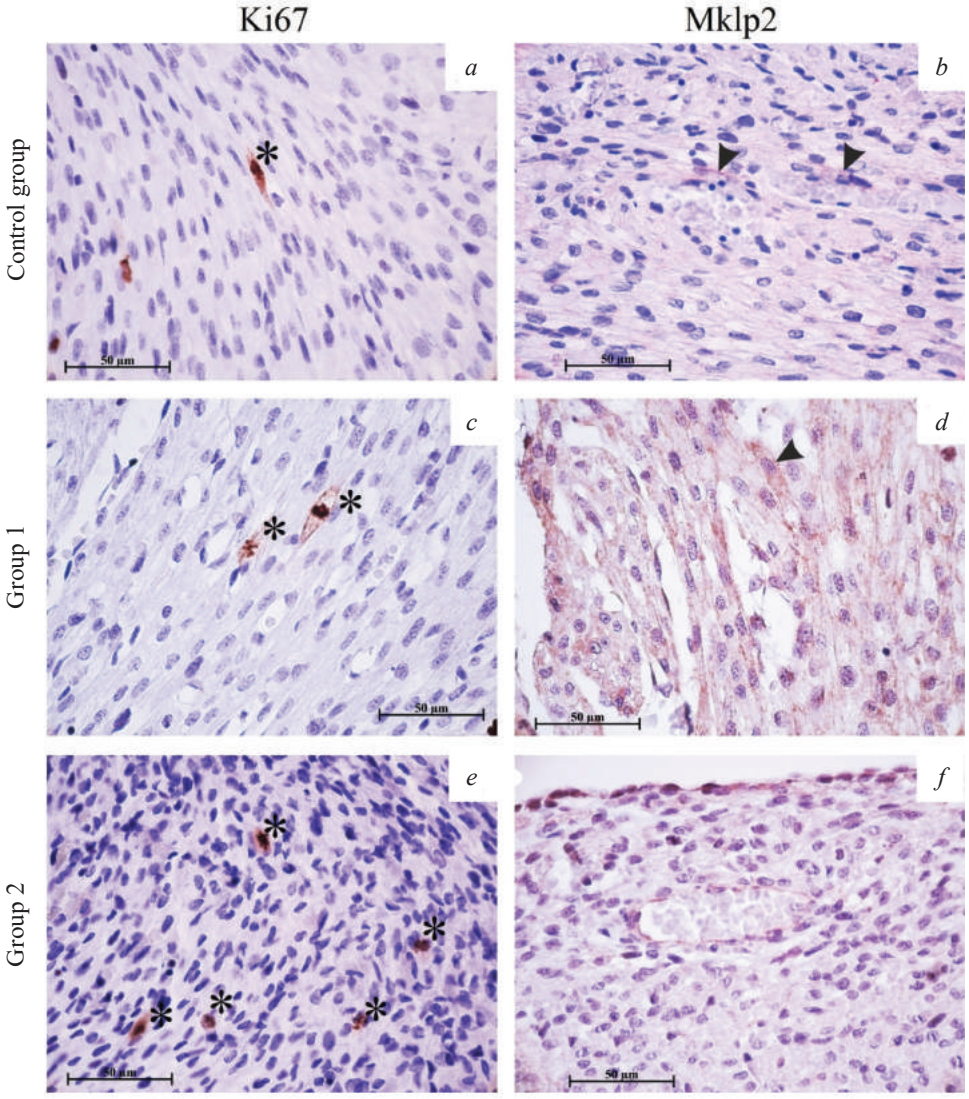

Figure. The left ventricular myocardium in rats: of the control group (full-term animals) (*a*, *b*), of group 1 – rats born on day 21.5 of gestation (*c, d*), of group 2 – rats born on day 21 of gestation (*e, f*), on day 1 of postnatal ontogenesis. Asterisks indicate Ki67-positive cardiomyocytes (*a, c, e*), and arrowheads indicate Mklp2-positive cardiomyocytes (*b, d, f*). Counterstaining with Gill's hematoxylin.

In the left ventricular myocardium of rats in all studied groups, the number of Ki67-positive cardiomyocytes exceeded the number of Mklp2-positive cardiomyocytes (Table 2). Changes in the number of Ki67-positive cardiomyocytes and Mklp2-positive cardiomyocytes per 1 mm^2 of the left ventricular section are shown in Table 2.

In all experimental groups, the number of Ki67-positive cardiomyocytes in the left ventricular wall of rats peaked on days 2–3 of postnatal ontogenesis, after which it progressively decreased. In the rats of group 1, a delayed increase in the number of Ki67-positive cardiomyocytes in the left ventricular wall was observed, compared with the control group. An increase in the number of Ki67-positive cardiomyocytes in the left ventricular wall of the rats from group 1 was found on days 3–5 of postnatal ontogenesis. On the contrary, in the rats of group 2, the number of Ki67-positive cardiomyocytes in the left ventricular wall exceeded that in the rats of group 1 and the control group on day 1 of postnatal ontogenesis.

The number of Mklp2-positive cardiomyocytes in the left ventricular wall of rats in all studied groups progressively decreased during the experiment. Preterm birth did not result in a change in the number of Mklp2-positive cardiomyocytes in the left ventricular wall of the rats.

Table 2

Changes in the number of immunopositive cardiomyocytes in the left ventricle of rats, number of cardiomyocytes in 1 mm^2, *Me* (Q_1; Q_3)

Group	Postnatal ontogenesis, day					
	1	2	3	4	5	6
Number of Ki67-positive cardiomyocytes						
Control group	43.8 (37.5; 50.0)	87.5 (68.8; 98.4)	68.8 (62.5; 85.9)	43.8 (25.0; 50.0)	46.9 (37.5; 50.0)	34.4 (20.3; 48.4)
	–	$p_2 = 0.000$	–	$p_2 = 0.001$	–	–
Group 1	28.1 (14.1; 42.2)	78.1 (68.8; 115.6)	96.9 (71.9; 123.4)	78.1 (57.8; 93.8)	62.5 (51.6; 75.0)	50.0 (37.5; 67.2)
	p =0.007	$p_2 = 0.000$	$p = 0.008$	$p = 0.000$	$p = 0.001$	
Group 2	71.9 (56.3; 81.3)	84.4 (75.0; 100.0)	65.6 (51.6; 95.3)	43.8 (37.5; 68.8)	46.9 (31.3; 62.3)	43.8 (31.3; 56.3)
	$p = 0.000$ $p_1 = 0.000$	$p_2 = 0.000$	–	$p_1 = 0.000$ $p_2 = 0.002$	–	–
Number of Mklp2-positive cardiomyocytes						
Control group	25.0 (0; 50.0)	12.5 (0; 50.0)	12.5 (0; 31.3)	0 (0; 25.0)	0 (0; 0)	0 (0; 0)
	$p_3 = 0.002$	$p_{33} = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$
Group 1	25.0 (0; 50.0)	0 (0; 31.3)	0 (0; 31.3)	0 (0; 25.0)	0 (0; 0)	0 (0; 0)
	p_3 =.009	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$
Group 2	12.5 (0; 31.3)	0 (0; 31.3)	0 (0; 25.0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$	$p_3 = 0.000$

Note: the level of statistical significance of differences in comparison with the control group (p), in comparison with group 1 (p_1), in comparison with the corresponding parameter for the previous period (p_2). The level of statistical significance of differences in the number of Ki67-positive and Mklp2-positive cardiomyocytes per 1 mm^2 of the left ventricular wall section in rats of similar groups at corresponding times (p_3).

DISCUSSION

The study used a generally accepted model of preterm labor induction in rats [21–23]. Before delivery, a decrease in the concentration of progesterone is observed in the rat blood plasma. It is known that morphological and functional changes in the uterus and placenta of rats caused by injection of mifepristone, a competitive progesterone antagonist, are identical to those developing before delivery in full-term pregnancy [21–23]. Mifepristone does not inhibit lactation [24], has no toxic effect, and does not cause stillbirth and infant death [18, 25, 26].

During the last day of prenatal ontogenesis, the mass of the rat heart increases from 15.8 to 25.9 mg [27]; the volume of the left ventricle increases from 4.14 to 6.72 mm^3 [28]. An increase in the size of the rat heart in prenatal and early postnatal ontogenesis is due to proliferation of cardiomyocytes. It was demonstrated that on days 1–2 of postnatal ontogenesis, an increase in the pool of cardiomyocytes (hyperplasia of cardiomyocytes) continues, which is consistent with the data of F.Li et al. [29]. It was shown that hyperplastic myocardial growth in postnatal ontogenesis mainly affects the subendocardial part of the myocardium. On days 3–4 of postnatal ontogenesis, rat cardiomyocytes lose the ability to complete cytokinesis: true mitosis is completely replaced by endomitosis (hypertrophy of cardiomyocytes). To assess whether preterm

birth will alter the time of myocardial transition from hyperplasia to hypertrophy, we studied the dynamics in the number of Ki67- and Mklp2-positive cardiomyocytes in preterm rats from day 1 to day 6 of postnatal ontogenesis.

Preterm birth is not accompanied by a compensatory increase in the number of Mklp2-positive cardiomyocytes or a change in the temporal pattern of Mklp2 expression in the rat left ventricular myocardium. An isolated increase in Ki67 expression without a change in Mklp2 expression by cardiomyocytes in the left ventricular wall of preterm rats indicates acceleration of cardiomyocyte hypertrophy, which may be the cause of a decrease in the myocardial flow reserve. An increase in Ki67 expression by cardiomyocytes of the left ventricular wall in preterm rats reflects the so-called catch-up growth and is an adaptive response of a structurally immature heart to an increase in the hemodynamic load due to the birth and growth of the animal.

It should be noted that an increase in the number of Ki67-positive left ventricular cardiomyocytes in rats born on day 21 of gestation, compared with the animals of the control group and the animals born on day 21.5 of gestation, is observed already on the first day of postnatal ontogenesis. Greater structural immaturity of the heart may be the cause of more pronounced morphological and functional changes in the organ following the birth. Taking into account short duration of the fetal stage of prenatal ontogenesis in rats (for Wistar rats, it lasts from day 18 to day 22 of embryogenesis) and high intensity of organogenesis during this period, it is likely that the 0.5-day difference in the duration of pregnancy can be the cause of a different effect of preterm birth on the morphological and functional condition of the rat myocardium. Further studies are required to understand in detail the causes of the observed differences in the effect of preterm birth on the proliferation of cardiomyocytes in rats born 12 and 24 hours before full term.

CONCLUSION

In early postnatal ontogenesis, a change in the pattern of Ki67 expression by cardiomyocytes in the rats born 12 or 24 hours before full term was revealed. An isolated increase in Ki67 expression without a change in Mklp2 expression by cardiomyocytes in the left ventricular wall of preterm rats indicates acceleration of cardiomyocyte hypertrophy. Shorter duration of the intrauterine development is associated with more pronounced morphological and functional changes in the rat myocardium.

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