2. E. G. Korvin-Pavlovskaya, A. S. Kul'minskaya, E. M. Karalova, et al., Tsitologiya, No. 2, 148 (1983).
3. Yu. A. Magakyan, Tsitologiya, No. 3, 243 (1976).
4. Yu. A. Magakyan and E. M. Karalova, Tsitologiya, No. 7, 888 (1978).
5. Yu. A. Magakyan and E. M. Karalova, Tsitologiya, No. 10, 1137 (1985).
6. A. W. Ham and S. H. Cormack, Histology, Philadelphia (1979).
7. B. E. Walcer, Texas Rep. Biol. Med., 17, 175 (1959).
8. E. Wolff and K. Konrad, Science, 174, 1034 (1971).

EFFECT OF HYPOXIA ON PRIMARY CARDIOMYOCYTE CULTURE

N. S. Stvolinskaya, Ts. I. Gerasimova, and B. F. Korovkin

UDC 616.127-008.922.1-008.64+616.127-005.14/-092.4+612.172:612.262/.06:612.273.2

KEY WORDS: cardiomyocytes; cells in culture; hypoxia.

The use of a culture of heart muscle cells as a model of states of hypoxia and ischemia of the myocardium has many advantages [1, 3]. The body responds to creation of ischemia by a multicomponent compensatory reaction.

In the investigation described below a state of hypoxia was modeled on a primary culture of neonatal rat cardiomyocytes in a constant-temperature gas-flow chamber, with the use of two gas mixtures containing 0 and 5% of oxygen respectively, and attempts were made to select conditions at which pathological changes in the cells were reversible in character.

EXPERIMENTAL METHOD

Primary cultures of neonatal rat cardiomyocytes were prepared from the hearts of 3-day-old rats. The hearts were minced, washed to remove blood, and incubated in a 0.1% solution of collagenase ("Sigma") in medium 199 at 37°C for 2.5 h. The material was then dispersed in a small volume of medium 199 by gentle pipetting. A solution of Versene (1:1) was added to the cell suspension thus obtained, and the mixture was centrifuged (800g, 3 min). The residue was resuspended in Eagle's medium with the addition of 10% serum (a mixture of different quantities of embryonic calf serum and bovine serum). The cells were seeded into Carrel's flasks in a concentration of not less than 1×10^6/ml. After incubation for 2 h at 37°C in an atmosphere containing 5% CO_2, about 50% of the cells settled on the glass. These flasks were discarded. The suspension of nonadherent cells was transferred into 12-well panels with coverslips. During the first 3 days the medium was changed daily, and thereafter every other day. Contracting cells, on reaching the state of a confluent monolayer, on the 4th-6th day of culture, were used in the experiments. The fraction of cardiomyocytes in the cultures was estimated in preparations stained for mitochondria by Mallory's method. Hypoxia and anoxia were produced in a constant-temperature gas-flow chamber by the use of two gas mixtures: 100% N_2 and 90% N_2 + 5% CO_2 + 5% O_2. The duration of exposure was 1 and 2 h at 37°C. The medium was changed 2 h before the experiment began. The state of the cells was assessed by light microscopy in preparations stained with Ehrlich's hematoxylin. To assess the relative proportions of the cells in culture, no fewer than 1000 cells were counted in each preparation under a magnification of 400. The average was found for four or five preparations taken from different experiments.

EXPERIMENTAL RESULTS

When obtaining a culture of cardiomyocytes there is the risk of contamination by fibroblast-like cells, which multiply much faster than cardiomyocytes, and during long-term culture they may displace all the other cell types in mixed cultures. Fibroblasts and endothelial

Research Institute of Medical Enzymology, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 106, No. 11, pp. 607-609, November, 1988. Original article submitted December 25, 1986.

0007-4888/88/0011-1624$12.50

TABLE 1. Characteristic Changes in Neonatal Rat Cardiomyocyte Culture during Experimental Hypoxia and Anoxia

Composition of gas mixture	Time, h	Contractility	% of cells with morphological changes	Contractility	Rehabilitation for 24 h (% of cells with morphological changes)
5% O_2, 5% CO_2, 90% N_2	1	—	9,65±1,45	+	0,56±0,05
	2	—	23,9±4,47	—	12,8±3,1
100% N_2	2	—	29,6±8,2	—	16,81±4,6

cells were separated from cardiomyocytes by taking advantage of their property of adhering to glass much more rapidly than cardiomyocytes [4]. High concentrations of cells also were used during seeding, the aim being to obtain a culture in a state of a confluent monolayer by the 4th-6th day in culture.

After 1 day of culture the cardiomyocytes adhere to the glass, spread out, and some of them resume their contractile activity (Fig. 1a). On the 4th day the cells reach the state of a confluent monolayer and begin to lie in a certain direction relative to each other, forming groups of cells making close contact and with the appearance of a contracted node (Fig. 1b). In each group the cells contract at the same rhythm (20-60 beats/min). Cultures in which, in each field of vision of the microscope under a magnification of 70, one large or several small contracting groups of cells were observed were used in the experiment.

In preparations stained by Mallory's method the cardiomyocytes and fibroblasts differed in the number of mitochondria. They were much more numerous in the cardiomyocytes, in which they were found in the form of a thin network in the cytoplasm. The cytoplasm in the fibroblasts was more homogeneous (Fig. 2). Cardiomyocytes in our preparations accounted for about 80% of the cells (79.04 ± 8.28%).

In the model of the state of hypoxia morphological changes in the cells were assessed in the preparations and the absence or presence of contractions was recorded; the state of the cell cultures after preliminary exposure to hypoxia, followed by culture under normal conditions for 24 h, also was investigated. During hypoxia and anoxia the cells lost their contractility. After rehabilitation for 24 h their contractility was restored if they had previously been exposed to hypoxia with a 5% O_2 concentration for 1 h, but in other cases it was not. The principal types of morphological changes in the cardiomyocytes were identical for all cases of experimental hypoxia (Fig. 3). These were vacuolation (Fig. 3a), evaginations on the cell surface and contracture of the cells (Fig. 3c), and detachment of the membrane from the glass (Fig. 3b). Multiple evaginations were found most frequently. This fact also was observed by other workers [5]. The quantitative results of assessment of the morphological changes are given in Table 1. This table shows that with a reduction in the O_2 concentration in the gas mixture and with an increase in the duration of exposure, there was an increase in the fraction of cells showing morphological changes and preserving them after rehabilitation. In all preparations many cells remained without morphological changes, but whose contractile activity was irreversibly lost.

The results show that anoxia and hypoxia with a 5% O_2 concentration and with an exposure of 2 h lead to irreversible loss of the contractile function of neonatal rat cardiomyocytes and irreversible morphological changes. Hypoxia with a 5% O_2 concentration and an exposure of 1 h causes reversible functional and morphological changes in the cardiomyocytes in a monolayer culture.

Various investigators have used models of hypoxia and cardiomyocytes in vitro to attempt to characterize cells: activity of various enzymes and their release into the external medium have been analyzed [1, 5, 6], and a fall of the ATP level has been described [2, 3]. In all cases the model was created in an atmosphere of pure N_2, and irreversible changes were obtained. Our experiments were begun with morphological studies of a cardiomyocyte culture incubated with two different gas mixtures — containing 0% and 5% of O_2, and this was followed by rehabilitation of the cells in a normal atmosphere, so as to be able to assess the reversibility of the disturbances arising. The results show that the use of a neonatal rat cardiomyocyte culture under conditions of hypoxia with a 5% O_2 concentration and an exposure for 1 h

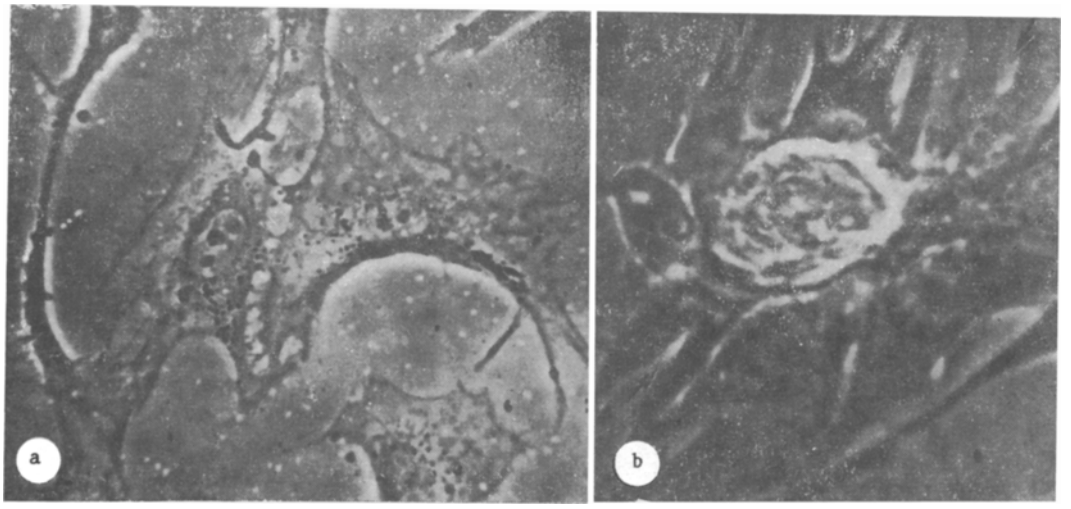

Fig. 1. Culture of neonatal rat cardiomyocytes: a) spreading out of cardiomyocytes on glass; b) formation of a group of closely contacting cells, adherent by their base to glass (arrows), free part of cells in close contact with one another and projecting above glass in the form of a contracting node. Phase contrast (400 ×).

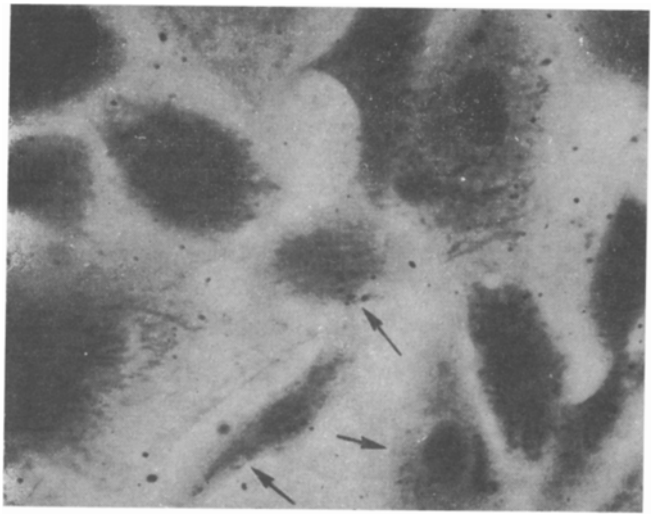

Fig. 2. Group of cells in monolayer culture. Stained by Mallory's method, fibroblasts indicated by arrows (400 ×).

provides a convenient model with which to investigate cell metabolism in reversible pathological states and to discover ways of correcting them.

In some investigations using models of anoxia and suspensions of cardiomyocytes the viability of the cells was estimated by their ability to excrete trypan blue (0.3%) [2]. If the cells lost their viability they began to be irreversibly stained with trypan blue in the region of the nucleus. We also attempted to use this test to assess viability of cardiomyocytes in culture. We found that if the experimental pathological state was reversible all 100% of cells preserved their ability to excrete trypan blue. If, however, the cardiomyocytes irreversibly lost their contractile function, ability to excrete trypan blue was preserved by more than 90% of the cells; consequently, ability to excrete trypan blue by cardiomyocytes in primary culture is not an unambiguous indicator that their specific function is preserved. In our opinion, preservation of the contractile function or reversibility of its loss in cardiomyocyte culture is evidence of the reversibility of the pathological process.

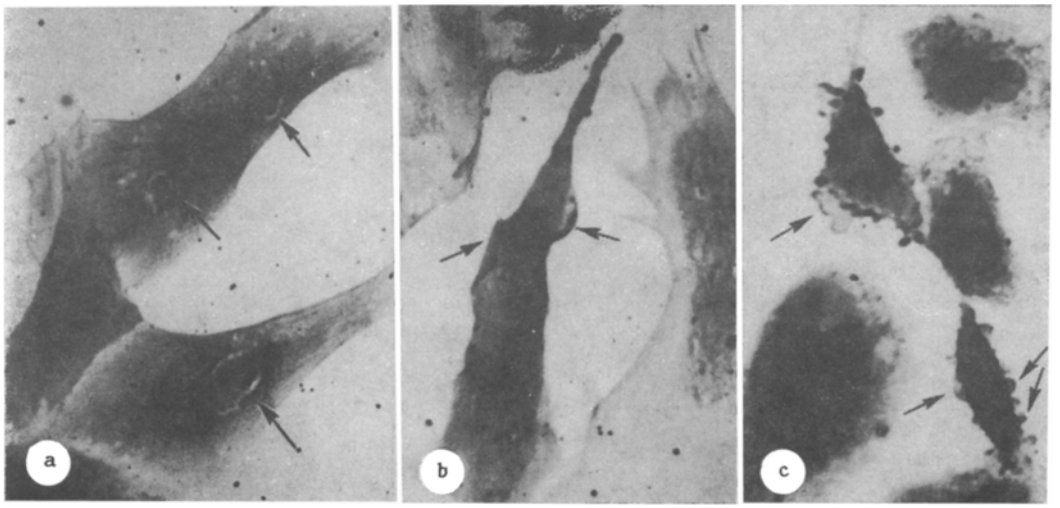

Fig. 3. Morphological changes in cardiomyocytes during experimental hypoxia. a) Gross vacuolation; b) detachment of membrane from glass; c) evaginations on cell surface and contracture of cells. Corresponding morphological changes indicated by arrows (400 ×).

LITERATURE CITED

1. D. Acosta, M. Puckett, and R. McMillan, In Vitro, 14, 728 (1978).
2. R. A. Altschuld, J. R. Hostetler, and G. P. Brierly, Circulat. Res. 49, 307 (1981).
3. E. Brussel, M. Freyss-Begun, G. Griffation, and P. Lechat, Biochem. Pharmacol., 34, 145 (1985).
4. F. H. Kasten, In Vitro, 8, 128 (1972).
5. H. M. Piper, P. Schwartz, R. Spahr, et al., J. Mol. Cell. Cardiol., 16, 385 (1984).
6. J. Rajs, J. Mol. Cell. Cardiol., 12, 1227 (1980).

EFFECT OF LONG-TERM HYPOKINESIA ON CIRCADIAN RHYTHM OF HEPATOCYTE PROLIFERATION

S. E. Li

UDC 616.36-018.1-02:612.766.2/-07

KEY WORDS: hypokinesia; proliferative activity; circadian rhythm.

Hypokinesia disturbs coordinated interaction between the muscular system, activity of the visceral systems of the body, and the level of their regulation [5-8, 12]. This results in changes in the temporal organization of the various physiological functions of the organs and to a disturbance of their circadian rhythms [2, 3].

The investigation described below was conducted in order to study proliferative activity of hepatocytes at different times of the 24-h period in rats kept under conditions of long-term hypokinesia.

EXPERIMENTAL METHOD

Male Wistar rats from the "Stolbovaya" nursery weighing 150-160 g were used. The total number of 38 animals was divided into two groups, one of which was kept under normal animal house conditions (control) whereas the other was exposed to hypokinesia in individual constraining

Department of Physiology and Pharmacology, Institute of Marine Biology, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. (Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 106, No. 11, pp. 610-611, November, 1988. Original article submitted December 25, 1987.