Organotypic tissue culture investigation of homocysteine thiolactone cardiotoxic effect

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Homocysteine thiolactone was demonstrated to inhibit the growth of 10–12-day-old chicken embryo cardiac tissue explants at 7 × 10^{-9}–1 × 10^{-3} М concentrations in a dose-dependent manner. The maximal cardiotoxic effect of homocysteine thiolactone was detected at 1 × 10^{-3} М, which corresponds to severe hyperhomocysteinemia. The results of experiments on culturing of cardiac tissue explants in the medium containing homocysteine thiolactone (1 × 10^{-3} М) and ouabain at concentrations regulating the signal-transducing (1 × 10^{-10} М) and pumping (1 × 10^{-8} М) functions of Na^+, K^+-ATPase indicate that the cardiotoxic effect of homocysteine thiolactone is supposed to result from inhibition of the Na^+, K^+-ATPase pumping function.

Keywords: organotypic culture, cardiac tissue explants, PlasDIC-contrast, homocysteine thiolactone, Na^+ , K^+-ATPase.

The changes in homocysteine and homocysteine thiolactone blood level can be regarded as markers of developing cardiovascular or neurological pathology. An increase in blood concentrations of these agents is especially harmful for pregnant women, as homocysteine produces a pronounced neurotoxic effect during the fetal life (1, 7, 9). Cardiac remodeling, which is characterized by accumulation of interstitial and perivascular collagen, was detected in hyperhomocysteineemic animals (2). The data about mechanisms of cardiac remodeling upon homocysteine action are controversial (11, 13).

The influence of homocysteine thiolactone on cell growth and proliferation in 10–12-day old chicken embryo cardiac tissue explants is studied in the present paper by organotypic tissue culture method.

Materials and Methods

Culture medium
pH 7.4: 45% – Hank’s solution; 40% – Eagle’s medium; 15% – fetal cow serum; supplemented with glucose (0.6%), insulin (0.5 ED/ml), gentomycin (100 ED/ml), glutamine (0.35%) (6).
Drugs
Homocysteine thiolactone (Sigma, USA) and ouabain (Sigma, USA) were added to the experimental Petri dishes. Control explants were cultured in standard culture medium.

Animal preparation
Experimental animals were 10–12-day-old White Leghorn chicken embryos provided by Sinyavino Poultry, Leningrad District, Russia. Cardiac tissue explants were obtained by extirpation from the ventricle of heart.

Organotypic tissue culture
Studies were performed on 800 explants. Cardiac tissue explants were cultured for three days on collagen supports in Petri dishes at 37 °C with 5% CO₂. CO₂-incubator (Sanyo, Japan) was used. Each Petri dish contained approximately 25 explants.

The growth of explants in tissue culture was controlled on vital preparations using a confocal laser scanning microscope LSM-710 (Carl Zeiss, Germany). The preparations were also visualized by a television microscope attachment Axiostar Plus (Carl Zeiss, Germany). Acridine orange (0.01%) was used to stain the explants.

Microscopic research methods
A laser confocal microscope (LSM-710, Carl Zeiss, Germany) was integrated with a standard Carl Zeiss Axio Observer Z1 inverted optical microscope. A PlasDIC-contrast was used for better visualization of organ culture growth in plastic Petri dishes.

Hill coefficient calculation
The cardiac tissue explants area index dependence on homocysteine thiolactone concentration was approximated by the Hill equation:

$$AI = 1 - \frac{1}{1 + \left(K_D/[HT]\right)^X},$$

where $AI$ is the area index value, $K_D$ – homocysteine thiolactone concentration inducing a 50% decrease in AI, $[HT]$ – homocysteine thiolactone concentration, $X$ – the Hill coefficient.

Statistical analysis
Cardiac tissue explants growth was quantified using the ImageJ program. The area index ($AI$) was used to estimate the explants growth. It was calculated in relative units as the ratio of total explant area to the central zone area. AI value was taken as 100% in the control experiments. The data were processed with STATISTICA 6.0 and Student’s $t$-test at $p = 0.05$.

Results
An intravital microphotograph of 10-day-old chicken embryo cardiac tissue explant made by a confocal laser scanning microscope LSM-710 (Carl Zeiss, Germany) using a PlasDIC-contrast is presented in Fig. 1. In the course of vital microscopic examination two zones could be observed in the explants after three days of culturing: the central zone (more dense) and the less optically dense peripheral zone, which surrounds the central zone. In both control and experimental explants the peripheral zone (the growth zone) comprised mostly proliferating cardiomyocytes (multinucleate polyploid cross-striated spindle-shaped cells, 65–70%), as well as fibroblasts (up to 35%) (Fig. 2). In some experiments a spontaneous contractive activity of cardiomyocytes was observed and registered by real-time photography.
Influence of homocysteine thiolactone on 10–12-day-old chicken embryo cardiac tissue explants growth was investigated at $7 \times 10^{-9} - 1 \times 10^{-3}$ M agent concentrations (Fig. 3). Upon addition of homocysteine thiolactone at $7 \times 10^{-9}$ M, a 13% decrease in AI of experimental explants was detected after three days of culturing. AI of experimental explants cultured in the medium containing homocysteine thiolactone at $4 \times 10^{-8}$ M was 26% less than the control value. A statistically significant inhibition (33% and 29%, correspondingly) of explant growth was observed upon addition of homocysteine thiolactone at $7 \times 10^{-7}$ and $8 \times 10^{-6}$ M (Fig. 3). At $1 \times 10^{-3}$ M homocysteine thiolactone inhibited the growth of explants by a mean of 60%, thus expressing a pronounced cardiotoxic effect. Homocysteine thiolactone did not exhibit any growth-stimulating effect on 10–12-day-old chicken embryo cardiac tissue explants.

The usage of a confocal microscope made it possible to perform a 3-D reconstruction of investigated explants, to optically register the formation of cell layers, to measure the
thickness of the forming growth zone, and to compare the data obtained in the pharmacological analysis with the control data. A new criterion to estimate the explant growth was introduced – the thickness of the growth zone (μm). This is the only approach to describe the synthetic processes occurring in the growth zone, which is formed layer-by-layer. It is necessary to note that so far the growth zone cells have been considered to form a monolayer (5). The thickness of the growth zone of cardiac tissue explants cultured in the medium with addition of homocysteine thiolactone did not differ from the control value at none of the tested concentrations of the agent and was equal to 22 μm.

Approximation of AI dependence on homocysteine thiolactone concentration by the Hill equation made it possible to estimate $K_D$ ($10^{-4}$ M). The Hill coefficient ($X$) was equal to 0.18, which might result from a negative cooperativity. Action of homocysteine thiolactone was monotonously dose-dependent. A cardiotoxic effect of homocysteine thiolactone was discovered. A statistically significant inhibition of cardiac tissue explants growth by homocysteine thiolactone was observed at $4 \times 10^{-8}$–$1 \times 10^{-3}$ M concentrations of the agent.

In order to elucidate the putative mechanism of homocysteine thiolactone cardiotoxic action, a separate set of experiments was carried out. Cardiac tissue explants were cultured in the medium containing homocysteine thiolactone at its inhibiting concentration of $1 \times 10^{-3}$ M together with ouabain at $1 \times 10^{-8}$ M or $1 \times 10^{-10}$ M.

In accordance with our prior data, ouabain (a selective inhibitor of Na⁺,K⁺-ATPase, $1 \times 10^{-10}$ M) stimulates the growth of cardiac tissue explants by an average of 37% (6). It is the concentration of ouabain that regulates the signal-transducing function of Na⁺,K⁺-ATPase.

Ouabain being applied at $1 \times 10^{-8}$ M inhibits the growth of cardiac tissue explants almost completely (6). AI value is equal to 5% of the control value. Ouabain at $1 \times 10^{-8}$ M inhibits both the signal-transducing and pumping functions of Na⁺,K⁺-ATPase.

AI of experimental explants cultured in the medium containing homocysteine thiolactone at $1 \times 10^{-3}$ M and ouabain at $1 \times 10^{-10}$ M was equal to 57% of the control value (Fig. 4). There were now observed the significant differences between the effects of homocysteine thiolactone ($1 \times 10^{-3}$ M) and homocysteine thiolactone ($1 \times 10^{-3}$ M) in phone ouabain ($1 \times 10^{-10}$ M). AI of the explants cultured in the presence of homocysteine thiolactone at $1 \times 10^{-3}$ M and ouabain at $1 \times 10^{-8}$ M was equal to that of the explants cultured in the medium containing only ouabain. Thus, it is likely that the cardiotoxic effect of homocysteine thiolactone results from inhibition of the Na⁺,K⁺-ATPase pumping function.

![Fig. 3. Cardiotoxic effect of homocysteine thiolactone](image-url)
Fig. 4. Combined effect of homocysteine thiolactone (1 × 10^{-3} M) and ouabain (1 × 10^{-10} M) on 10–12-day-old chicken embryo cardiac tissue explants growth

**Discussion**

Apart from a negative effect on the cardiovascular system, an elevated concentration of homocysteine in the blood plasma provokes the development of neural tube defects (1), abnormal pregnancy (7) and neurodegenerative diseases (3). Hyperhomocysteinemia in humans also provokes, in particular, epileptic seizures (convulsions). Convulsive (8 mmol/kg) and subconvulsive (5.5 mmol/kg) homocysteine dosage was used in the development of models for testing the activity of various anticonvulsive drugs (8).

Inhibition of Na⁺,K⁺-ATPase activity upon hyperhomocysteinemia was detected using the experimental epilepsy model (3, 12). A study on Na⁺,K⁺-ATPase kinetics has demonstrated that homocysteine inhibits the pumping function of the enzyme (3, 12). Homocysteine effect on the Na⁺,K⁺-ATPase signal-transducing function was not investigated.

Homocysteine thiolactone inhibits cardiac tissue explants growth in a dose-dependent manner, as demonstrated in the current paper. The maximal cardiotoxic effect of homocysteine thiolactone was achieved at 1 × 10^{-3} M. This concentration corresponds to severe hyperhomocysteinemia. AI of experimental explants was 60% lower than the control value.

A putative reason for cardiac remodelling upon homocysteine action could be an increase in the number of mast cells which release mediators inducing cardiomyocytal hypertrophy, cell death or fibrosis (4, 10). Application of the organotypic tissue culture method made it possible to estimate the direct influence of homocysteine thiolactone on a cardiac tissue cell community. The responses of the immune system, which should be taken into consideration upon in vivo administration of the agent, were thus eliminated. Results of cardiac tissue explants culturing in the medium containing homocysteine thiolactone (1 × 10^{-3} M) and ouabain at effective concentrations that regulate the signal-transducing (1 × 10^{-10} M) and pumping (1 × 10^{-8} M) functions of Na⁺,K⁺-ATPase indicate that the cardiotoxic effect of homocysteine thiolactone presumably results from inhibition of the Na⁺,K⁺-ATPase pumping function.
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