Cardiac Fibroblasts Mitochondrial Activity in the Presence of Medicinal Preparations with Various Pharmacological Effects

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Abstract

The studies of cardiac fibroblasts mitochondria activity using fluorescence probe MitoTracker Red CMXRos (Thermo Fisher Scientific, USA) and proliferation capacity with MTT assay (Thermo Fisher Scientific, USA) were carried out under the influence of antihypoxant (trimetazidine) and anticancer (doxorubicin, cytosar) medicinal preparations. It was revealed that medicinal preparations from the antitumor antibiotics group decreased mitochondria activity regardless of the cell state unlike antitumor metabolites. Medicinal preparation trimetazidine increases cardiac fibroblasts mitochondrial activity.

Keywords: Cardiac Fibroblasts, Mitochondria, MTT Assay, Antitumor Agent, Antibiotic, Antimetabolite, Antihypoxant, Cytotoxicity.

Background

Currently, preclinical studies at the molecular [1-3], cellular [4-7], organ [8, 9], organism levels [10-14], along with bioequivalence studies, therapeutic equivalence, and safety are an integral part of the development of new medicinal preparations [15, 16].

It is known that heart function is regulated via interaction of two main types of cells: cardiomyocytes (CMs) and cardio fibroblasts (CFs).

Heart fibroblasts are located in the layers of cardiomyocytes and play an important role during physiological functioning of the organ and at pathological states (hypertension, myocardial infarction, heart failure) [17, 18].

Because of cardiac fibroblasts specific localization these cells could activate reparative regeneration processes in myocardium during its damage due to increasing of paracrine activity and maintaining of extracellular matrix (ECM) homeostasis [19-24, 31].

At the same time, positive effect of treatment during myocardial damage directly depends on the correct pharmacotherapy strategy, which is aimed at both activation and inhibition of the activity of cardiac fibroblasts.

Energy saving effect of trimetazidine, cytoflavin, phosphocreatine and meldonium during experimental myocardial ischemia was revealed [25], whereas in cute heart failure trimetazidine played an important role in preventing myocardial fibrosis [26, 32].

Thus, study of morph functional state of cardiac fibroblasts under the influence of various medicinal preparations is an important to understand heart path physiology when safe and effective methods of drug therapy are developing.

Aim

The aim of the research is to study cardiac fibroblasts mitochondria activity under the influence of antihypoxant and anticancer medicinal preparations.

Material and Methods

Experimental researches with laboratory animals were carried out in accordance with international requirements [27]. Cardiac fibroblasts were isolated from newborns 6 day old rats' hearts ventricles.

The tissue was incubated in enzymes solution (trypsin/collagenase Type I, Thermo Fisher Scientific, USA). The cells released from the tissues fragments due to repeatedly pipetting.

The cells were cultured in 25 cm2 culture flasks (SPL Life Sciences Co., Ltd., Korea) in culture medium DMEM/F12 (Thermo Fisher Scientific, USA) containing 10% fetal calf serum (Biosera, France) and gentamicin (50 μg/mL) in CO2-incubator at 37°C, 100 % humidity and 5% CO2.

After 7 days of cultivation the cells were detached using trypsin and the number of cells in suspension was counted.

Then the cells were placed in the wells of 24-well plate (SPL Life Sciences Co., Ltd., Korea) in the amount of 3.5x105 cells per well and in the wells of 96-well plate (SPL Life Sciences Co., Ltd., Korea) in the amount of 5x104 cells per well.

Plate's wells with DMEM/F12 culture medium (Thermo Fisher Scientific, USA) without serum were used as positive control, wells with DMSO solution (cat. 85190 Thermo Fisher Scientific, USA) at a concentration of 5% were used as negative control.

During the research the following medicinal preparations were used: doxorubicin (Teva Pharmaceutical Industries, Ltd., Israel, 60 mg/m2) – antitumor anthracycline antibiotic which exhibits high antimitotic activity; cytosar / cytarabine (PFIZER, Inc., USA, 100 mg/m2) – antitumor antimetabolite, DNA synthesis inhibitor; trimetazidine (Teva Pharmaceutical Industries, Ltd., Israel, 60 mg/4,8 L) – antihypoxant, which improves oxygen utilization and reduces the need for it.

All the medicinal preparations were added into culture medium DMEM/F12 (Thermo Fisher Scientific, USA) which did not contain serum.

After 24 hours of cultivation medicinal preparations were added to cells which were growing in the wells of 24-well plate and cultured during 24 hours.

Then fluorescent dye MitoTracker Red CMXRos (Thermo Fisher Scientific, USA) at a final concentration of 200 μM was added to the wells, cells were cultured during 30 minutes in CO2-incubator.

The fluorescent dye accumulates in mitochondria due to mitochondrial membrane negative charge, and because of the presence of chloromethyl group, the dye binds to the thiols of proteins and peptides in the mitochondrial matrix.

Intensity of mitochondria fluorescence was studied with fluorescence microscope ECLIPSE Ti-S (NIKON, Japan) and software Nikon EZ-C1 FreeViewer (NIKON, Japan) in 10 visual fields.

Also, after 24 hours of cultivation medicinal preparations were added to cells which were growing in the wells of 96-well plate and cultured during 24 hours. Then 20 μL of MTT reagent (3-(4, 5dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide, cat. M6494, Thermo Fisher Scientific, USA) was added into each well.

The cells were incubated with the reagent during 2 hours in the conditions of CO2-incubator at 5% CO2 and 37^{0}C. At the end of incubation period the plate was removed from CO2-incubator, the solution was aspirated from the wells and DMSO solution (cat. D12345 Thermo Fisher Scientific, USA) was added. The plate was carefully shaken to dissolve formazan crystals.

Optical density of solutions was measured using ELISA photometer MultiskanFC (Thermo Fisher Scientific, USA) at 540 nm (reference absorbance value was 620 nm). The survival rate of cardiac fibroblasts was calculated using formula (1):

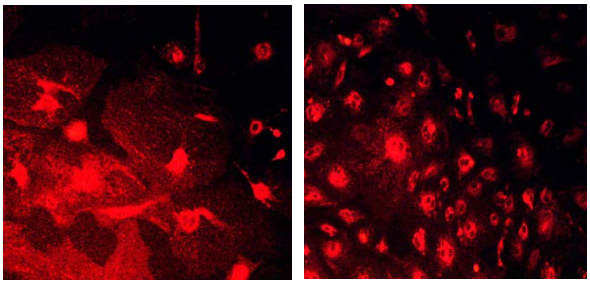
$$\frac{(OD\ experimental\ \ wells - OD\ medium\ )}{(OD\ control\ \ wells - OD\ medium\ )} \times 100\%, \quad (1)$$

Where OD – optical density.

Statistical data processing was performed using software Statistica 6.0.

Results and Discussion

During the research low mitochondria fluorescence intensity was revealed in the positive control group 51.52±10.02 a.u. in comparison with the negative control group 57.98±11.85 a.u., which possibly could be connected with the achievement by the cells "plateau" stage at which the cell life processes slow down due to impossibility of further proliferation due to the lack of nutrients and free space in the well, while cell morphology was not changed (Fig. 1).

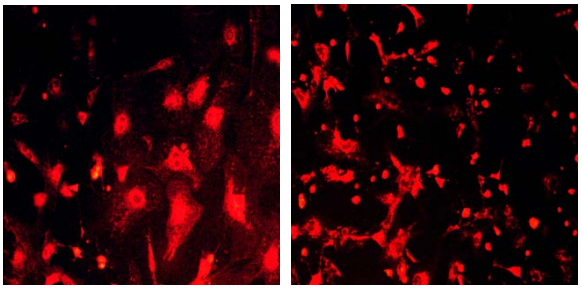


A B

Figure -1: Positive control group (A) and negative control group (B) cardiac fibroblasts fluorescence intensity. Magnification X 100.

Decrease of fluorescence intensity was revealed in the group of cells where doxorubicin was added – 53.24±9.61 a.u. The differences are not significant in comparison with positive and negative control groups, which probably are connected with the cytotoxic effect of the preparation.

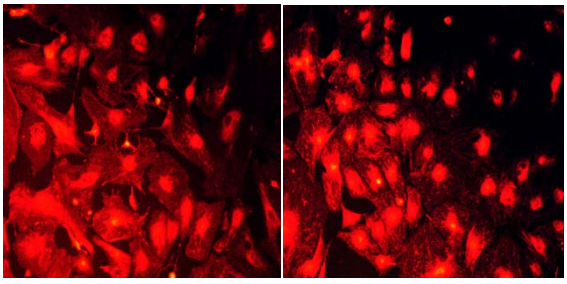
The morphology of the cells was greatly changed, there were cells detached from the bottom of the well (Fig. 2). The mechanism of cytotoxic effect of anthracycline antibiotics is mainly associated with nucleic acids synthesis inhibition by intercalation between pairs of nitrogenous bases, damaging of secondary helical DNA due to interaction with topoisomerase II, as well as binding with cell membrane lipids, accompanied by changes in ion transport and cellular functions.



A B

Figure- 2: Positive control group (A) and group with doxorubicin adding (B) cardiac fibroblasts fluorescence intensity. Magnification X 100.

The opposite effect was discovered in groups with adding of cytosar and trimetazidine medicinal preparations. Fluorescence intensity increased to 110.116 ± 24.24 a.u. and to 117.72±19.35 a.u. respectively, the differences were significant at $p \leq 0.01$ in comparison with both positive and negative controls, the morphology of cells was not changed (Fig. 3). Cytostatic effect of cytosar was associated with impaired synthesis of nucleic acids (DNA, RNA). Being a phase-specific antimetabolite cytosar predominantly acts in the S-phase of the cell cycle in which DNA replication occurs. Trimetazidine has a cytoprotective effect due to increasing of energy potential, activation of oxidative decarboxylation and rationalization of oxygen consumption (enhancement of aerobic glycolysis and blockade of fatty acid oxidation). Cytosar did not show cytotoxic effect, because the cells were at a "plateau" stage and did not divide. As an antimetabolite it possibly triggered the processes which were connected to activation of thiol groups of proteins and peptides in mitochondrial matrix. Increasing of fluorescence intensity during trimetazidine application is associated with glycolysis activation within the cell.



A B

Figure- 3: Group with cytosar (A) and group with trimetazidine adding (B) cardiac fibroblasts fluorescence intensity. Magnification X 100.

The highest survival rate of cardiac fibroblasts was revealed for the cells incubated with culture medium which contained cytosar – 82.86%, the differences were significant in comparison with negative control group ($p \leq 0.01$). Also, the high survival rate of cells was revealed for cardiac fibroblasts cultured with trimetazidine – 80%, the differences were significant in comparison with negative control ($p \leq 0.01$). When the cells where incubated with doxorubicin it accompanied by their low survival rate – 57,14%, the differences were significant in comparison with positive control ($p \leq 0.01$) (table 1).

Table -1: Optical density of the experimental samples

No of Group	The Name of Probe	Optical density, a.u.
1	Blanks	0.018±0.002
2	Positive control group	0.053±0.008
3	Negative control group	0.037±0.005 *
4	Wells with culture medium containing cytosar	0.047±0.01 #
5	Wells with culture medium containing doxorubicin	0.038±0.005 *
6	Wells with culture medium containing trimetazidin	0.046±0.007 #

* – differences are significant in comparison with positive control group ($p \leq 0.01$); # – the same with negative control group ($p \leq 0.01$).

It is known that MTT assay is based on an ability of mitochondrial dehydrogenases to convert water soluble 3-(4, 5dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide into formazan, which crystallizes inside the cell [22, 23, 24]. The obtained results of MTT assay conforms with the results of mitochondrial activity study with fluorescence probe MitoTracker Red CMXRos. Cytosar and trimetazidine stimulate mitochondrial activity due to activation of intracellular metabolic processes, while doxorubicin inhibits both mitotic activity and intracellular metabolic processes.

Conclusions

1. Medicinal preparations from the antitumor antibiotics group decrease mitochondria activity regardless of the cell state unlike antitumor metabolites.

2. Medicinal preparation trimetazidine increases cardiac fibroblasts mitochondrial activity.

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