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Research Article

# Effect of cyclophosphamide on regulation of heart contractions by means of sodium calcium exchanger

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## Abstract

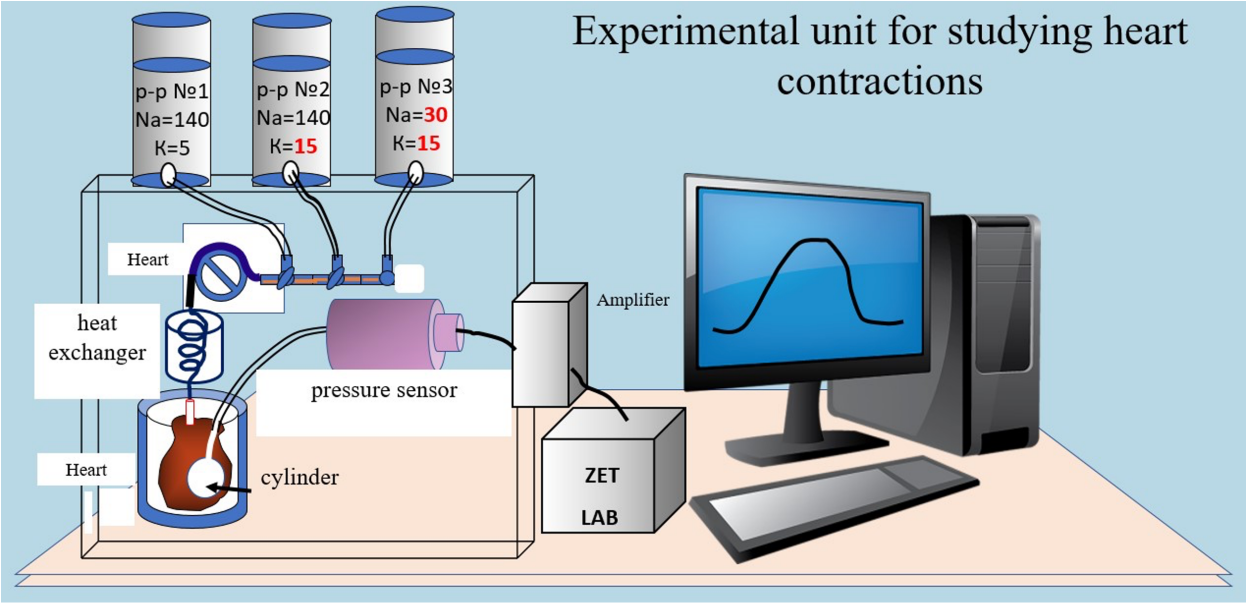
**Introduction:** Every year brings in new medications capable to slow or stop proliferation of tumour cells. Unfortunately, in spite of antitumour benefits, new medicines have some side effects that reduce their therapeutic properties.

**Materials and Methods:** The study was conducted in three series of experiments on 24 white Wistar rats, both male and female, each weighing 200-250 g. Decapitation of the animal was performed under ether anesthesia with rapid extraction of the heart and perfusion using the Langendorff method. To study $Na^+/Ca^{2+}$ exchanger (NCX) in the heart, a perfusion device was used to create a permanent coronary flow. Monitoring the physiological state of the heart when changing the composition of solutions was carried out using a balloon inserted into the left ventricle. Contractions and relaxation of the heart were recorded using an electronic pressure sensor. The parameters were documented and processed using the Zet Lab external module software.

**Results and Discussion:** In the first series of experiments, the effect of hyposodic solution on the tone of the left ventricle of the heart stopped by a hyperpotassic medium was studied. The developed technique served as the basis for studying the effect of cyclophosphamide on NCX, accompanied by contraction and relaxation of the heart. Experiments have shown the ability of cyclophosphamide to significantly reduce the rate of the tone increase and the development of contraction force, as well as prolong the relaxation time during NCX.

**Conclusion:** Cyclophosphamide is able to disrupt the capture of ionized calcium in the cytosol by intracellular Ca-accumulating structures during relaxation of the heart. Unlike control recordings, in the presence of cyclophosphamide, repeated relaxations do not occur completely. As a result, each subsequent contraction begins at a higher initial diastole level.

## Graphical abstract




## Keywords

$Na^+/Ca^{2+}$ exchanger, slow calcium channels, sarcoplasmic reticulum, cyclophosphamide, isolated rat's heart contractions

## Introduction

The variety of medicines is rapidly increasing due to the globalization of the pharmaceutical market. Every year, there is a growth of an innovative resource that transforms the clinical practice, the course and the outcomes of diseases. Concurrently, modernized methods are being developed and introduced into the healthcare practice. Stratified medicine is rapidly being incorporated into the era of scientific and technological breakthrough, characterized by the genotyping of malignant neoplasms and targeted medicines. This is due to the use of real clinical practice data jointly with the facilities of artificial intelligence. Current achievements of drug therapy are annually supplemented with new promising medicines, including those that effectively slow down or inhibit tumor activity. Nevertheless, having an advantage in antitumor activity compared to others, new medicines have a number of side effects that reduce their therapeutic properties (Gorvin 2019; Abulfadl et al. 2023).

However, the combination of cytostatics with biologically active agents in some cases makes it possible to weaken the negative effects of such drugs, like in experiments with doxorubicin, which, in addition to its cytostatic properties, causes a cardiodepressive effect. This cytostatic was found to have the ability to weaken the activity of the transport carrier, which controls the intracellular level of Ca ions by blocking the $Na^+/Ca^{2+}$ exchanger (NCX) (Hilgemann 2020; Al-Khannaq and Lytton 2022; Bomfim et al. 2024).

In this regard, it is of particular interest to study the effect of other cytostatics on NCX, in order to find new ways to weaken their cardiodepressive effect on the heart. Cyclophosphamide was chosen as a prototype due to its cytostatic effect as well as its ability to disturb a contractile function of the heart (Taslimi et al. 2019).

According to the publications, the cardiotoxic effect of this cytostatic is manifested in the form of a progressive decrease in the left ventricular pressure and reduced myocardium contraction and relaxation rates (Ferreira de Souza et al. 2019; Liu et al. 2020; Mourouzis et al. 2023).

In this regard, in this study, an attempt was made to investigate the effect of cyclophosphamide on the contractile activity of the heart, regulated exclusively by NCX.

This system of regulation of heart contractions attracts special attention by its direct participation in the electromechanical coupling of the myocardium. Cardiac contraction is a multi-stage process in which calcium ions play a major role. In the final stage, these cations are released from the sarcoplasmic reticulum, causing an interaction between actin and myosin. In this case, the contraction force depends on the amount of calcium released from the reticulum. The initiator of calcium release from the reticulum is a small portion of calcium getting inside cardiomyocytes through calcium channels during electrical excitation of the heart.

The second source of calcium activating the reticulum is NCX, which is able to increase intracellular calcium concentration by exchanging sodium for extracellular calcium. Action potentials in cardiomyocytes are known to be accompanied by a rapidly incoming sodium flow during the opening of sodium channels. In turn, the increase in sodium ions inside the cells, in the primembrane layer, causes their exchange for external calcium ions. As a result, an additional amount of calcium taking part in the activation of the sarcoplasmic reticulum and contraction of the heart appears (O'Halloran 2020; O'Donnell and Jones 2023).

Thus, in addition to the participation of slow calcium channels, NCX is an additional participant in initiating the process of heart contraction. Under physiological conditions, these two mechanisms seem to work synchronously. However, until now, the role of the transmembrane exchanger in muscle contraction has not been fully studied (Xue et al. 2023).

In order to study the regulation of contractions, exclusively with the help of NCX, it is necessary to conduct experiments under conditions of elimination of other sources of calcium intake into cells. It is known that the inactivation of the sodium and calcium channels can be carried out using a high extracellular concentration of potassium. It is important that at the same time there is a complete cessation of heart contractions. Therefore, under these experimental conditions, cardiac contractions can occur only due to NCX.

Thus, the purpose of this study was to determine the possibility of NCX involvement in contractions and relaxation of the isolated heart, as well as to investigate the ability of cyclophosphamide to influence this process.

# Materials and Methods

### Animals

The experiments were carried out on white Wistar rats (Ethics Committee of Voronezh State Medical University named after N.N. Burdenko – Minutes №5 dated September 19, 2023) in strict compliance with the current requirements of The Rules of Laboratory Practice for Experimental (Preclinical) Research in the Russian Federation (GOST 351000.3-96 and 51000.4-96). In total, 24 male and female rats weighing 200-250 g were used in the study. One series of experiments was conducted on 10-12 rats, depending on the reproducibility of the research results. The animals were decapitated under ether anesthesia. The heart was quickly removed and placed in a cooled perfusion solution. Cardiac perfusion was performed using the Langendorff method (Frolova et al. 2020).

### Models

To study the state of NCX in the heart, a perfusion device that creates a constant flow rate of nutrient solution through the heart was used, under control of pressure in the aorta, which ranged from 60-70 mm Hg. Solution No.1 was introduced into the aorta of the isolated heart at a rate of 8 mL/g of raw weight of the following composition: NaCl – 140 mM, $NaHCO_3$ – 2 mM, KCl – 5 mM, TRIS-OH – 5 mM, (pH 7.4), $CaCl_2$ – 2 mM, and glucose – 11 mM. The solution was saturated with oxygen and heated to a temperature of 36 C.

The experiments were started after stabilization of metabolic processes and contractile function of the heart with solution No.1 within 10 minutes.

After that, heart contractions were stopped using solution No.2. The solution contained all the same components as solution No.1 but was different from it by the concentration of KCl that was three times increased. Solution No.2 had the following components: NaCl – 140 mM; $NaHCO_3$ – 2 mM; KCl – 15 mM; Tris-OH – 5 mM (pH 7.4); $CaCl_2$ – 2 mM; and glucose – 11 mM. The solution was saturated with oxygen and

heated to a temperature of 36 °C.

The high concentration of potassium chloride caused depolarization of cardiomyocytes. At the same time, the electrical and contractile activity of the heart completely stopped (Fig. 1).

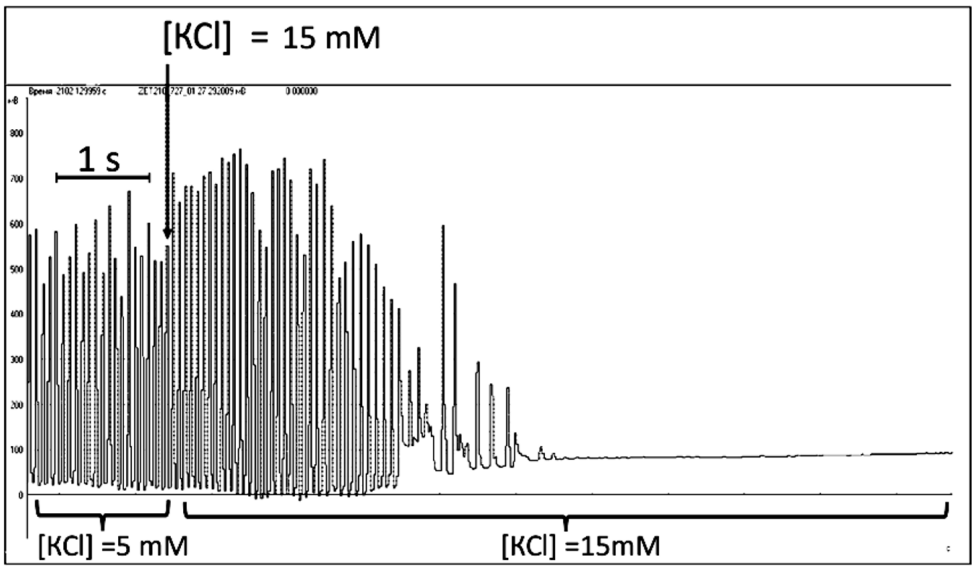



**Figure 1.** Cardiac arrest in a rat caused by an increased potassium level to 15 mM in perfusion solution №2.

Five minutes after perfusion with solution No.2, the sodium-dependent absorption of calcium ions by the heart was stimulated with solution No.3. This solution, unlike solution No.2, was characterized by a lower (80%) concentration of sodium chloride. The composition of the solution was as follows: NaCl – 30 mM; mannitol – 220 mM; $NaHCO_3$ – 2 mM; KCl – 15 mM; Tris-OH – 5 mM (pH 7.4); $CaCl_2$ – 2 mM; and glucose – 1 mM. The solution, like all previous ones, was saturated with oxygen and heated to a temperature of 36 °C.

The lack of osmotic pressure in perfusion solution No.3, as a result of a decrease in the concentration of sodium chloride, was compensated by the introduction of mannitol into a hyposodic solution. Since mannitol, unlike NaCl, does not dissociate into separate particles, its concentration was doubled compared to the concentration of the deficient sodium chloride. Meanwhile, the amount of mannitol in solution No.3 was 220 mM. It can be seen from the above list of solution No.3 components that the concentration of KCl in the hypotonic solution still remained high – 15 mM.

In comparison with solution No.2, the ratio of concentrations between sodium and calcium ions in solution No.3 was changed. The predominance of calcium levels over sodium ions on the outside of cardiac cells was accompanied by a predominant interaction of calcium with $Na_+$-$Ca_{2+}$ carrier. As a result, calcium ions were transferred inside the cells in exchange for intracellular sodium (Yue et al. 2020).

While doing that, 6 indicators were calculated:

1. The increase rate in the tone of the heart left ventricle during perfusion with solution No.3 (V rising tension);
2. The relaxation rate during perfusion with solution No.2 ($V_{relaxation}$);
3. The maximum heart contraction force at the beginning of perfusion with solution No.3 ($H_1$);
4. The heart contraction force after 5 minutes of perfusion with solution No.3 ($H_2$);
5. The time from the initiation of NCX to the maximum of muscle contraction ($T_1$ rising);
6. The time from the beginning to the complete muscle relaxation ($T_2$ relaxation).

At the end of perfusion with a hyposodic medium (solution No.3), solution No.2 having, as indicated above, a higher content of potassium chloride and the absence of mannitol was passed through the heart.

The physiological state of the heart, during the change of solutions, was monitored using a ballon inserted into the left ventricle. Pressure changes were recorded using an electronic pressure sensor. The signals from the sensor were sent to an analog-to-digital converter. Using the Zet Lab external module software, the parameters were recorded and processed using a computer (Fig. 2).

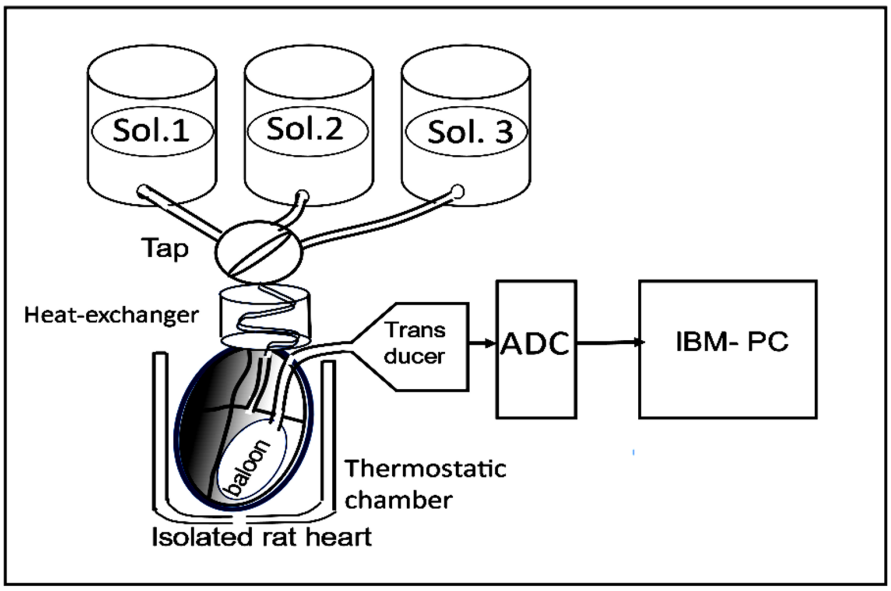



**Figure 2.** The unit for recording heart contractions with the help of NCX.

It is known that the process of NCX in forward and reverse (reverse) modes is carried out periodically with each contraction of the heart. Therefore, we simulated the transition from one state of the heart to another by repeating cycles of reducing extracellular sodium concentration and returning it to its previous concentration on the same heart preparation with 5-minute breaks. After each experiment, the heart was dried with filter paper and weighed.

**Compounds under study**

Reagents marked as chemically pure (Russia) were used to prepare the solutions. Cyclophosphamide of pharmaceutical production, manufactured in powder form, was used as a cytostatic agent. Cyclophosphamide was added 100 mg per 1 liter of each perfusion solution. Meanwile, the final concentration of cytostatic in solutions was $5 \cdot 10^{-4}$ M. As a rule, such concentrations are used to study the effect of medicinal substances on animal organs.

**Statistical processing**

All the data obtained were checked for probability distribution andб in the case when the sample corresponded to a normal distribution, statistical data processing was carried out using the Student's t-test. All calculations were performed in the computing environment of the Statistica tabular processor (StatSoft Inc., USA).

# Results

The experiments began with perfusion of the heart with solution No. 1 for 10 minutes to stabilize biochemical processes and physiological state. Then the contractions of the heart were stopped with solution No. 2, in which the potassium chloride content was increased three times – to 15 mM. After 5 minutes, the experiments began in which cardiac perfusion was performed with solution No. 3, in which the concentration of sodium chloride was reduced to 30 mM. At the same time, the amount of potassium chloride still remained high (15 mM).

Experiments have shown that a decrease in the extracellular concentration of sodium ions from 140 mM (solution No.2) to 30 mM (solution No.3) was accompanied by an increase in intraventricular pressure in the heart stopped by a hyperpotassic medium (Fig. 3). The maximum contraction force during NCX (H1) reflected the final result of NCX, in which the release of sodium from cardiomyocytes was accompanied by an increase in the concentration of calcium ions in the cytosol. Meanwhile, after 100 seconds from the beginning of perfusion with hyposodic solution, the contraction force in control experiments reached 63 mmHg (Table 1).

**Table 1.** The effect of cyclophosphamide on the process of muscle contraction associated with NCX in the rat's heart

| Indicators (units of measurement) | Control n=8 | Cyclophosphamide (1st recording) n=8 |
|---|---|---|
| V (contraction) the rate of the tone increase (mm Hg/s) | 1.31±0.02 | 0.27±0.01 p<0.001 |
| V (relaxation) the rate of )relaxation (mm Hg/s) | 0.98±0.03 | 0.91±0.05 p>0.5 |
| force of heart contraction after 100 seconds of perfusion with hyposodic medium (mm Hg) | 63±1.3 | 26±1.3 p<0.01 |
| force of heart contraction after 200 seconds of perfusion with hyposodic medium (mm Hg) | 48±1.1 | 44±2.5 p>0.5 |
| force of heart contraction after 300 seconds of perfusion with hyposodic medium (mm Hg) | 47±0.6 | 48±3.3 p>0.5 |
| time from NCX initiation to the maximum of muscle contraction (seconds) | 74±2.1 | 231±12 p<0.001 |
| time from the beginning of relaxation to complete relaxation of the muscle (seconds) | 130±2.7 | 143±5.1 p>0.05 |

***Note:*** *n* is the number of animals in each series of experiments.

An important result of the study was the discovery of the reversibility of the observed process. Following the contraction, it was possible to induce muscle relaxation, also with the help of a NCX. The transfer of cardiac perfusion to solution No.2 containing a physiological concentration of NaCl (140 mM) caused gradual relaxation of the muscle after the contraction stage. At the same time, the diastolic tension of the heart muscle reached an initial low level (Fig. 3). In the first series of experiments, the hearts of 8 animals were used.

Thus, the obtained results of preliminary experiments served as a clear proof of the direct participation of the NCX mechanism in the contraction and relaxation of the heart muscle. In addition, the developed technique made it possible to begin studying the effect of cyclophosphamide on NCX-reaction associated with reduction–relaxation.

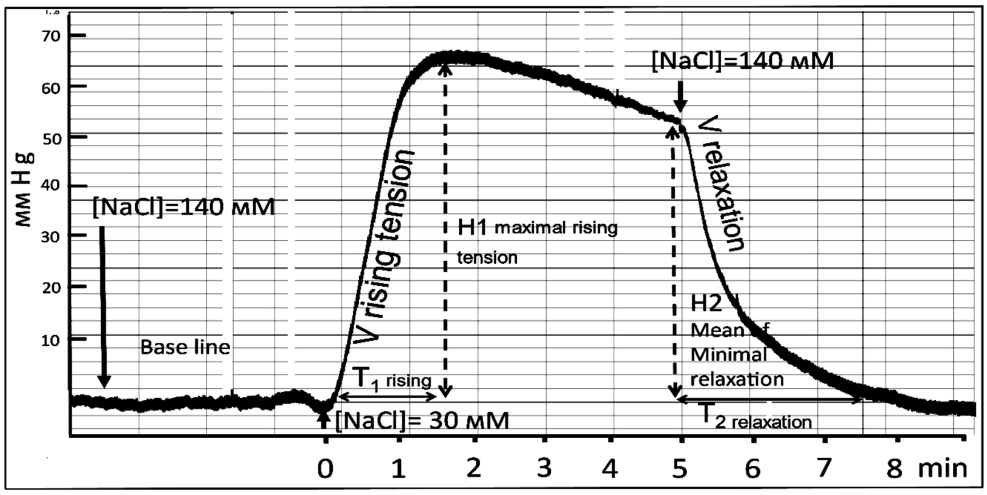



**Figure 3.** Changes in pressure in the left ventricle of the rat's heart during activation of NCX by reducing extracellular the sodium level is up to 30 mM. ***Note:*** The abscissa axis indicates the muscle tension in mmHg. The ordinate axis indicates the time in minutes.

In the next series of studies on 8 animals, the effect of cyclophosphamide on NCX induced contraction and relaxation was studied. A record of one of the eight responses is shown in Figure 4.

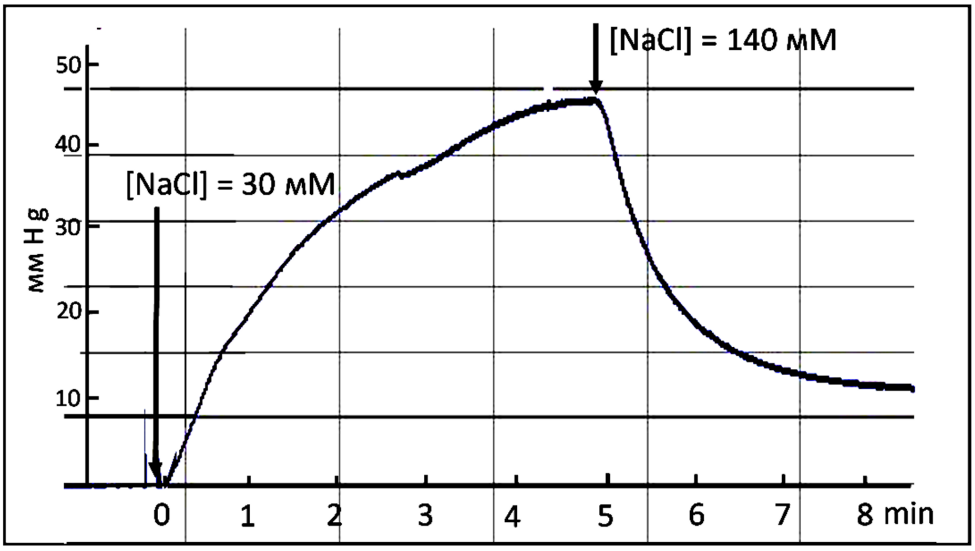



**Figure 4.** The effect of cyclophosphamide on heart contraction and relaxation in rats induced by NCX. ***Note:*** The abscissa axis indicates the muscle tension in mmHg. The ordinate axis indicates the time in minutes. Designations: [NaCl] = 140 mM – perfusion with solution No.2. [NaCl] = 30 mM – perfusion with solution No.3.

It can be seen that, unlike control records, the reduction process under the influence of low sodium levels proceeds at a slow rate. The increase in tone was completed only 5 minutes after the start of perfusion with hyposodic solution. Relaxation of the heart muscle after changing the hyposodic solution (30 mM) to the normosodic solution (140 mM) occurred with approximately the same intensity as in the control. The mathematical processing of the experimental results is presented in Table 1.

Comparison of the experimental results with the indicators of the control records demonstrates the significant effect of cyclophosphamide on the calcium flow into cardiomyocytes during activation of NCX process. Attention is drawn to a significant slowdown at the rate of the tone increase, a sharp decrease in the force of contraction, immediately after the initiation of NCX. In this regard, the time from NCX initiation to the maximum of muscle contraction slows down. For greater visualization, the differences between control and cytostatic experiments are shown in Figure 5.

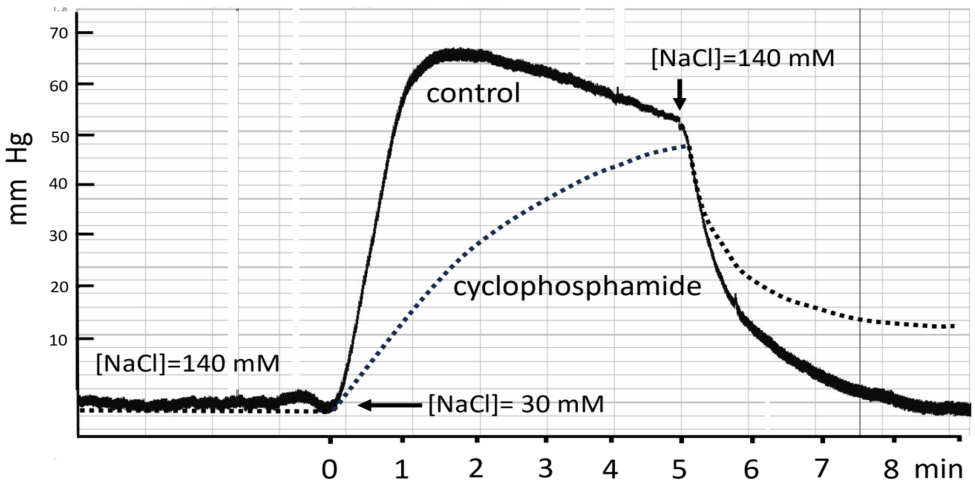



**Figure 5.** Comparison of the dynamics of the rat's heart tone increase in the control and in the presence of cyclophosphamide in a reaction medium. ***Note:*** The abscissa axis indicates the muscle tension in mmHg. The ordinate axis indicates the time in minutes. Designations: [NaCl] = 30 mM – perfusion with solution No.3; [NaCl] = 140 mM – perfusion with solution No.2.

It should be noted that the process of NCX is weakened by cyclophosphamide by certain adverse factors. Among them, several can be assumed: firstly, damage to the Na-Ca exchange mechanism that ensures the transfer of Ca into cells. Secondly, there is a violation of the process of calcium release from the sarcoplasmic reticulum, which has lost the ability to respond to portions of calcium penetrating into the myoplasm during Na-Sa metabolism. The third possible option is to change the properties of the actomyosin complex, which is not able to respond to an increase in intracellular calcium levels.

It should be noted that in presence of cyclophosphamide, NCX is hindered by some unfavourable factors, several of which are possible. The first one is damage to NCX that ensures Ca transfer into the cells. The second possible obstacle is blocking the sarcoplasmic reticulum, which has lost the ability to respond to portions of calcium entering the myoplasm during NCX. And the third possible unfavourable factor is the change in the properties of the actomyosin complex, which is not able to respond to the increase in intracellular calcium level.

In the second series of experiments, heart contractions were not limited to only NCX stimulation. The application of a hyposodic solution was performed on the same heart three times. It was believed that if the damage to the carrier was significant, then the repetition of the initiating stimuli, with the help of NCX, should lead to the same results as with the first stimulus. The results of these experiments are shown in Figure 6 and Table 2.

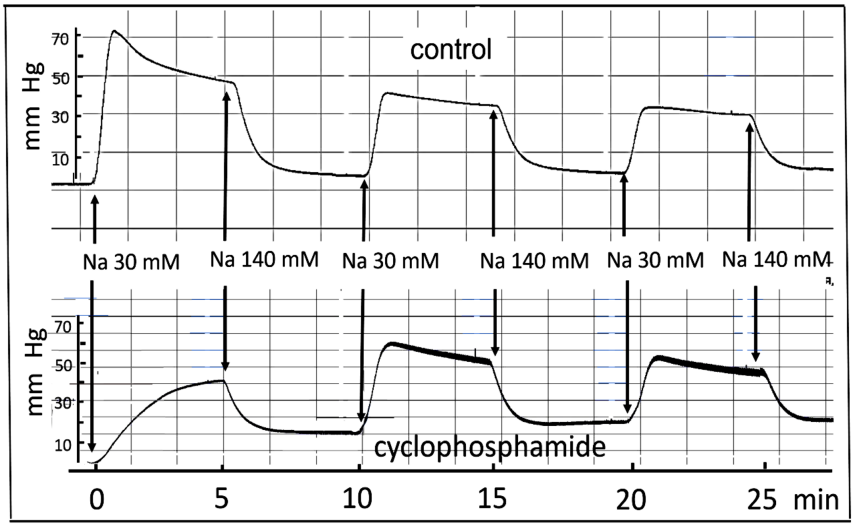



**Figure 6.** Recordings of contractions and relaxation of the rat's heart caused by triple activation of NCX. ***Note:*** The abscissa axes indicate the muscle tension in mmHg. The ordinate axes indicate the time in minutes. Designations: [NaCl] = 140 *mM* – perfusion with solution No.2. [NaCl] = 30 *mM* – perfusion with solution No.3.

From the above visual demonstration, it can be seen that cyclophosphamide has a noticeable effect on the parameters of contraction and relaxation of the heart only during the first cycle of initiation of these processes with NCX.

Repeated decreases and increases in the concentration of sodium chloride caused, as usual, contractions and relaxation of the heart muscle. However, the rate of contractions and relaxation of the muscle, under the influence of cytostatics, significantly increased. Consequently, from the second time of initiation of contractile activity of the heart, NCX continued to actively direct calcium ions into the myocardial cells. The other parameters differed little from the control indicators. Therefore, the first assumption about the possibility of damage to the transport carrier by cyclophosphamide was not confirmed.

The third suspected cause of changes in the kinetics of cardiac contraction under the influence of cytostatics associated with damage to the actomyosin complex was not been confirmed, either. The presence of cyclophosphamide did not decrease, but on the contrary, increased the rate of contraction $V_{(contraction)}$, compared with the control (Table 2). This observation indicates the maintenance of a sufficient power of the heart muscle contractile apparatus.

To answer the question about the possibility of a calcium ion balance disturbance in the cytoplasm of the muscle during contraction and relaxation, the tests were complicated in the third series of experiments. The number of repetitions of NCX was increased to 6 times. In total, 8 more animals were used in this series of experiments. Since the results

of the experiments with the 1st, 2nd and 3rd repetitions turned out to be almost identical to previous experiments (Table 2), the indicators of contractions and relaxation during the 4th, 5th and 6th repetitions are presented in the form of diagrams in Figure 7.

In experiments with cyclophosphamide, it was found that at the 4th repetition, cytostatic has a negative effect on the rate of relaxation of the left ventricle of the heart (Fig. 7A). At the same time, the rate of the tone increase practically did not change.

**Table 2.** The effect of cyclophosphamide on repeated heart contractions caused by NCX

| Indicators (units of measurement) | the 2nd repeat | | the 3rd repeat | |
|---|---|---|---|---|
| | Control n=8 | Cyclophos-phamide n= 8 | Control n=8 | Cyclophos-phamide n=8 |
| V (contraction) the rate of the tone increase (mm Hg/s) | 1.25±0.03 | 1.60±0.06 P<0.05 | 1.20±0.04 | 1.66±0.06 P<0.05 |
| V (relaxation) the rate of relaxation (mm Hg/s) | 0.94±0.03 | 1.26±0.05 P<0.05 | 0.91±0.03 | 1.07±0.3 P<0.05 |
| force of heart contraction after 100 seconds of perfusion with hyposodic medium (mm Hg) | 58±0.9 | 59±1.7 P>0.5 | 59±1.1 | 56±1.5 P>0.5 |
| force of heart contraction after 200 seconds of perfusion with hyposodic medium (mm Hg) | 48±1.06 | 51±1.6 P>0.5 | 48±1.0 | 45±1.3 P>0.5 |
| force of heart contraction after 300 seconds of perfusion with hyposodic medium (mm Hg) | 77±1.2 | 72±1.9 P>0.5 | 72±1.5 | 66±0.7 P>0.5 |
| time from NCX initiation to the maximum of muscle contraction (seconds) | 125±3.2 | 120±3.9 P>0.5 | 117±2.4 | 118±3.9 P>0.5 |
| time from the beginning of relaxation to complete relaxation of the muscle (seconds) | 1.25±0.03 | 1.60±0.06 P<0.05 | 1.20±0.04 | 1.66±0.06 P<0.05 |

***Note:*** n is the number of animals in each series of experiments.

To a large extent, under the influence of cytostatics, the force of heart contraction weakened after 100 and 300 seconds, during perfusion of the heart with hyposodic medium (Fig. 7B and 7C).

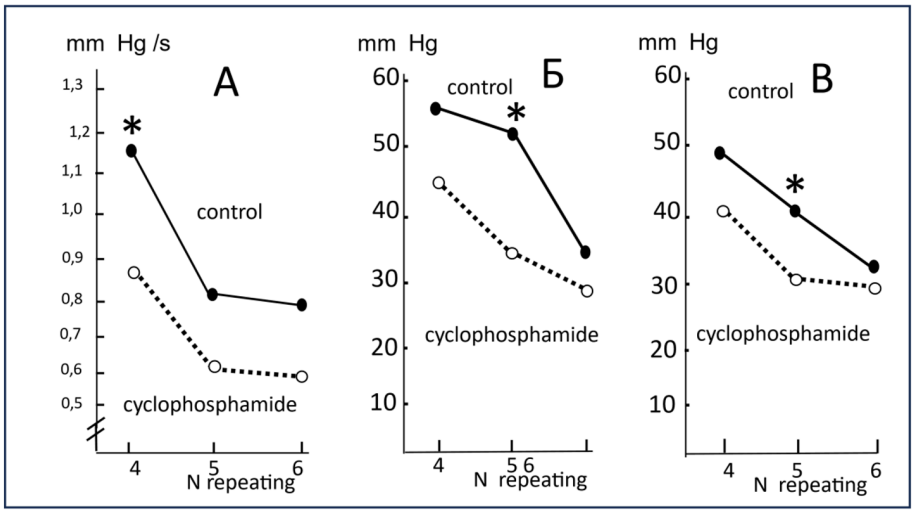



**Figure 7.** The effect of cyclophosphamide on the rate of muscle relaxation (A), on the force of cardiac contraction after 100 seconds (B) and the force of cardiac contraction after 300 seconds of perfusion with hyposodic medium (C) at 4th, 5th and 6th stimuli using NCX. *Note:* A continuous line shows indicators in the control, an intermittent line shows indicators in the presence of cyclophosphamide. The data of 8 experiments on the same heart are presented. * shows significant differences between the control and the experiment (cyclophosphamide) (p<0.05).

A decrease in the rate of relaxation and the force of contraction of the heart indicates a slowdown in the process of removing calcium from the cytoplasm of cardiomyocytes after each contraction cycle. Such a condition is possible only with an excess of free calcium in the cytosol, which fails to be deposited in a timely manner by the intracellular structures during the contraction completion.

# Discussion

Summing up the results of the study, it can be concluded that cyclophosphamide, apparently, disrupts the functioning of Ca-accumulating intracellular structures, possibly the sarcoplasmic reticulum. This opinion is based on the peculiarities of the muscle relaxation kinetics during the change of solutions from hyposodic to normosodic.

In the presence of cyclophosphamide, in contrast to control experiments, repeated relaxation was not performed completely (Figs 4 and 6). Subsequent contractions began at a higher initial diastole level.

This suggests that already before the first stimulation of NCX, before the onset of contractions, the cytostatic increased the level of free calcium in the cytoplasm of the cells. It is possible that the cytostatic has the selective property of reducing the activity of Ca-pumps in the membranes of the sarcoplasmic reticulum. Without affecting the transport carrier, without having a significant effect on the contractile apparatus of the muscle, the cytostatic caused an overflow of the cytosol inside cardiomyocytes with ionized calcium.

# Conclusion

Cyclophosphamide has the property of disrupting the reuptake of calcium ions by intracellular calcium-binding structures of cardiomyocytes. The resulting excess of calcium in the myoplasm creates conditions for maintaining a constant high muscle tone, which serves as an obstacle to the development of the maximum amplitude of heart contractions. This condition is known in medicine as ‘incomplete diastole’.

# Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

This research was approved by the meeting of the Ethics Committee of Voronezh State Medical University named after N.N. Burdenko – Minutes №5 dated September 19, 2023.

### Data availability

All of the data that support the findings of this study are available in the main text.

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