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Further analysis of rat myocardium contractility changes associated with a subchronic lead intoxication

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ABSTRACT

A moderate subchronic lead intoxication was observed in male rats after repeated intraperitoneal injections of lead acetate. Right ventricular trabeculae and papillary muscles were isolated for *in vitro* studying of the contraction-relaxation cycle under isotonic and physiological loading. The contractile function of the myocardium was also assessed by measuring the velocity of thin filament movement over myosin. Lead intoxication led in papillary muscles to a decrease in the maximal rate of isotonic shortening for all afterloads and a decrease in the thin filament sliding velocity. Papillary muscles from lead-exposed rats displayed marked changes in most of the main characteristics of afterload contraction-relaxation cycles, but in trabeculae these changes were less pronounced. The reported changes were attenuated to some extent in rats treated with a Ca-containing bioprotector. The amount of work produced by both types of heart muscle preparations was not changed by lead. Only in papillary muscles the load-dependent relaxation index was significantly increased in the lead-treated groups. Thus subchronic lead intoxication affects the peak rate of force development and relaxation properties of cardiac muscle contracting in isotonic/physiological regimes rather than the total amount of mechanical work, which may reflect adaptive changes in the myocardial function under decreased contractility.

1. Introduction

Lead has been known to affect humans since long ago. Its impact first resulted from occupational exposure to lead in metallurgy and lead-using technologies and then from contacts with lead in daily life (consumed with food cooked or kept in glazed earthenware or tinplated copperware and with drinking water supplied by lead pipes, which are still used in some water supply systems). In the recent centuries, the adverse effects of lead have been associated also with polymetallic contamination of the areas around a growing number of major industrial sources of lead-containing emissions, particularly copper smelters (Katsnelson et al., 2008). This “globalized” hazard of lead increased dramatically in the mid-20th century in connection with the use of leaded gasoline as engine fuel. The belated ban on its use imposed by many countries has not resolved the problem due to the high persistence of lead contamination in soil and biota.

Chronic lead intoxication affects nearly all organs and tissues in the organism (see e.g. reviews IPCS, 1995; Privalova, 2005 and many others), which makes it a particularly adverse factor. In this context, it is very important to explore connection between cardiovascular morbidity and occupational and environmental lead exposures as highly probable but still insufficiently evidence-based. In particular, there is scanty and rather contradictory information on the phenomenology and mechanisms of lead effects on the contractile function of the myocardium (Kopp et al., 1980a,b; Prentice and Kopp, 1985; Chao et al., 1984, 1990; Richardt et al., 1986; Sharma and Purohit, 2014; IPCS, 1995; Yang et al., 2017 Vassallo et al., 2008; Fioresi et al., 2013, 2014; Silva et al., 2015); please also refer to our recent paper which summarized the current findings on this issue (Protsenko et al., 2018). It also should be noted that previously reported *in vitro* studies concerned lead effects on the myocardial contractility were limited to *isometric* mode of contraction (corresponding to the isovolumic phase of the

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cardiac systole) while the main in vivo function of the whole heart is the ejection of the blood (the amount of stroke volume and velocity of the ejection/refilling).

In the present study, we provide for the first time equally important results obtained in the same experiment but related to muscle shortening in the *isotonic* and *physiological* modes of contraction corresponding to the ejection phase of the natural cardiac cycle. Since in a real cardiac cycle papillary muscles and trabeculae are experiencing unequal loads, we deemed it reasonable, as well as in the previous paper, to juxtapose the toxic effects of lead on corresponding preparations. Moreover, it is also for the first time that we obtained some experimental data revealing an effect of lead intoxication on the mechanical characteristics of actin-myosin interaction.

2. Materials and methods

Our experiments were carried out on outbred white male rats from our own breeding colony with an initial body weight of ca. 300 g. The rats were housed in conventional conditions, breathed unfiltered air, and were fed standard balanced food. The experiments were planned and implemented in accordance with the "International guiding principles for biomedical research involving animals" developed by the Council for International Organizations of Medical Sciences (1985) and were approved by the Ethics Committee of the Ekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers.

2.1. Animal model of subchronic lead toxicity

The text which follows reproduces virtually verbatim Sub-Section 2.1 of our previous study (Protsenko et al., 2018). However, it is essential in the present paper as well, to ensure that the reader has a clear idea of the experimental lead intoxication conditions under which we carried out the cardiotoxicity tests described below.

For inducing an explicit, even if moderate, non-lethal subchronic intoxication, a lead acetate solution was administered to male outbred rats with an initial age of about four months by intraperitoneal (IP) injections three times a week (up to 15 injections) at a one-shot dose of 12.5 mg of Pb per kg body mass. Four groups of 10 rats each were exposed in parallel to: sterile distilled water IP (control), lead acetate solution IP (Pb), the same solution IP along with oral administration of calcium (ca.150 mg Ca per rat daily) plus vitamin D3 (Pb + Ca), and oral administration of the same calcium plus vitamin D preparation alone (Ca).

Immediately after the end of the exposure period, the following procedures were performed for all rats:

- Weighing of the body.
- Estimation of the CNS ability to evoke temporal summation of subthreshold impulses (a variant of the withdrawal reflex and its facilitation by repeated electrical stimulations in an intact, conscious rat) (Rylova, 1964; Varaksin et al., 2014; Panov et al., 2015; Minigalieva et al., 2015, 2017);
- Recording of the number of head-dips into the holes of a hole-board which is a simple but informative index of exploratory activity frequently used for studying the behavioral effects of toxicants and drugs (e.g. Abeyemi et al., 2006; Fernandez et al., 2006; Varaksin et al., 2014; Panov et al., 2015; Minigalieva et al., 2015, 2017);
- Collection of daily urine for analysis of total coproporphyrin and δ-aminolevulinic acid (δ-ALA).

Blood was collected from the tail vein under ether anesthesia a week before the animals were sacrificed. The biochemical indices determined in the blood included total serum protein, albumin, globulin, alanine- and aspartate-transaminases (ALT, AST), triglycerides, cholesterol, high and low density lipoproteins, and calcium level. For determining the hemoglobin content, hematocrit, thrombocrit, mean erythrocyte volume and for counting RBC, WBC and thrombocytes, we used a MYTHIC-18 auto-hematology analyzer (C2 Diagnostic, Montpellier, France). Reticulocyte percentage was counted on smears under optical microscopy after supravital staining with brilliant cresyl blue. Cytochemical determination of succinate dehydrogenase (SDH) activity in blood lymphocytes was based on the reduction of nitrotetrazolium violet to formazan, the number of granules of which in a cell was counted under immersion microscopy. All the clinical laboratory blood and urine tests with the exception of the above ones were performed using the well-known techniques described in many manuals (e.g. Tietz, 1995).

Before euthanasia, ECG was recorded with the help of a medical three-channel small-size electrocardiograph EC3T 01 RD (OOO NPP « Monitor») from a lead similar to the standard lead II for humans. It provided reliable estimates of the heart rate and the QRS-complex amplitude only.

Then the rats were killed by cervical dislocation. The liver, spleen, kidneys, heart and brain were weighed. The whole heart was weighed before the dissection of myocardial strips from right ventricle.

2.2. Contractility of isolated myocardial preparations

For the same reasons as outlined in the beginning of the previous sub-section, we believe it necessary to reproduce partially the text of Sub-Section 2.2 from the paper by Protsenko et al. (2018).

The experiments were performed in a modified Krebs-Henseleit solution containing (in mM): NaCl 118.5; $NaHCO_3$ 14.5; KCl 4.2; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 11.1, $CaCl_2$ 2.5; with aeration by 95% O_2 + 5% CO_2, pH = 7.4 at a temperature of 30^{O}C. Fifteen minutes before killing the rats by cervical dislocation, the animals of all four groups were administered heparin (1000 ME per animal by intramuscular injection). The heart was removed immediately upon euthanasia and immersed into a Petri dish with normal saline with 2,3-butanedione monoxime (30 mM) for 15 min before dissection. Thin trabeculae and papillary muscles (dia. 300 μm, maximum) were dissected from the right ventricle[1] and mechanically fixed to the rods of the control and measurement device (one end to the force transducer and the other end to the length servomotor) in a thermostatic bath perfused by normal saline. The muscles were excited with ~5 ms overthreshold rectangular stimuli at a frequency of 1 Hz through non-polarizing carbon electrodes. The isometric-isotonic mechanical activity of the muscles was recorded by a two-channel setup and a Muscle Research System (Scientific Instruments GmbH, Heidelberg, Germany), both allowed us to record and control the contraction force and muscle length to an accuracy of ± 1 mg and ± 1 μm, respectively, and apply various pre- and afterloads to the muscle.

In the control program, the value of the afterload was set as fraction of the isometric force developed by muscle at given preload. During the twitch the program monitored the value of the developed isometric force every 100 μs. Upon reaching the level of afterload, the muscle actively shortened while its force kept equal to the afterload forming force plateau (Fig. 1A, upper and lower panels). The phases of isometric contraction and isotonic shortening (Fig. 1A, phases (a) and (b)) were the same for isotonic and physiological modes of contraction. Starting from peak systolic shortening, the further changes in force/length were different in isotonic *vs* physiological modes. In isotonic mode, the muscle was relaxed and returned to the initial diastolic length; thereafter the muscle continued relaxation at this constant length isometrically (Fig. 1A, phases (c) and (d)). In physiological mode, the muscle length was kept at the minimal systolic value. This provided isometric relaxation at end-systolic length, which lasted until the

[1] Left ventricular papillary muscles are too thick, which leads to hypoxia of innermost cardiomyocites during *in vitro* testing.

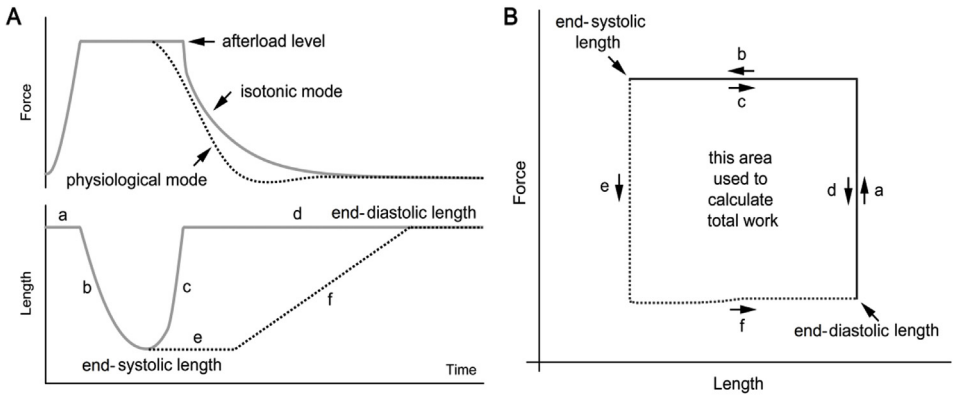

Fig. 1. An elucidative diagram on the difference between isotonic and physiological modes of contraction of cardiac muscle. (A) The curves of force (upper panel) and length (lower panel) obtained for isotonic (solid lines - phases a,b,c,d) and physiological (dotted lines - phases a,b,e,f) modes of contraction with equal afterloads. The particular phases of the twitches are as follows: a – isometric contraction at the initial length until the developed force is higher than the level of afterload, b – isotonic contraction (shortening) under a constant afterload, c – isotonic relaxation (lengthening back) under the same constant afterload, d – subsequent isometric relaxation ater phase c at the initial length that is also end-diastolic one; e – isometric relaxation at the end-systolic length (for the physiological mode) until the muscle force decreases to the given preload level at this new length, f – the length is returned to the initial (i.e. end-diastolic) position by the servomotor. The phases (a) and (b) are the same for isotonic and physiological modes. (B) Approximate scheme of the force-length loop representing isotonic and physiological modes of contraction. In both modes, the loop starts from the initial length (that is also end-diastolic one) and progresses through phases (a) and (b) until end-systolic length as indicated by arrows. In isotonic mode, the relaxation process then develops in the exact opposite way (c and d) thus forming a loop with zero area (the total work is 0). In physiological mode, the relaxation follows from the end-systolic point through phases (e) and (f) and this makes rectangular-shaped loop with non-zero area (the total work can be calculated as a square of the area under the force-length loop).

muscle force decreased to the given preload level at this new length. Then the isometric relaxation was stopped and the muscle returned by servomotor to the initial length. At this final moment it did not produce active force (Fig. 1A, phases (e) and (f)). This mode of contraction simulates the consecutive phases of in vivo ventricular contraction (isovolumic rise of pressure, ejection against aortic pressure, passive filling under low ventricular pressure). The advantage of this mode is that one is able to construct a force-length loop (Fig. 1B) which is conceptually the same as a pressure-volume loop for a whole heart. Moreover, the isotonic mode of contraction does not provide a tool for assessment of total work performed by the muscle during a twitch whereas this total work can be easily retrieved from force-length loop constructed for the muscle subjected to physiologically relevant sequence of loading (Fig. 1B).

Measurement of the mechanical response and a pattern of mechanical loading of papillary muscles and trabeculae isolated from the same heart were implemented in real time with the help of analogue-to-digital conversion of transducer signals and digital-to-analogue conversion of control signals from the computer through an ADC/DAC (PCI-1716S, AdLink Technology Inc., Taiwan). The real-time mode was supported by our own software operating within a real-time HyperKernel subsystem (Arc Systems Ltd., Japan) integrated with OS Windows XP. This software and hardware system enabled us to gather information and control the peripherals at 10 kHz sampling rate.

To ensure data comparison, the recordings obtained for the myocardial preparations of rats from the experimental groups were processed as follows:

- the force of the preparation was normalized to its cross-section area while the amplitude of shortening was normalized to its length at a given preload (95% L_{MAX} in our case);
- the maximum shortening velocity was calculated and normalized to preparation length, and then a force-velocity curve was constructed using a series of recordings under various afterloads;
- the recording of preparations' mechanical activity in the physiological loading mode enabled us to use the force and shortening trajectories, analogues of pressure and volume parameters, for constructing a phase profile, an analogue of the pressure-volume loop (Hisano and Cooper, 1987; Mast and Elzinga, 1990).
- the work performed by muscles in the physiological mode under a sequence of loads was estimated by the tension-length loop area for various afterloads (Suga, 1979; Loiselle et al., 2008; Han et al., 2013). To compare the data from various groups, force was normalized to cross-section area while length was normalized to length L_{MAX}, given the dimensionality of work (Work = tension*dL/L_{MAX}) to be (mN/mm^2)*(mm/mm).

The experiments were performed for a muscle length of 95% L_{MAX}, at which the myocardium produced maximum work (Layland et al., 1995). In accordance with the commonly accepted approach (Layland et al., 1995), the contribution of the passive force component to work production was not taken into account.

2.3. Statistical processing

If data for each of the groups displayed normal distribution, the significance of the differences was estimated by the Fisher analysis of variance. For statistical processing of the results we used the ANOVA multivariate analysis of variance (Statistica 10.0, StatSoft Inc.). The results are presented as mean ± SEM. We used nonparametric Mann-Whitney U test for multiple comparisons, and Student's t-test or Wilcoxon test for paired comparisons, differences were considered to be statistically significant at $P < 0.05$.

2.4. Assessment of actin–myosin in vitro interaction's mechanical characteristics

According to the theory of sliding filaments (Huxley and Niedergerke, 1954), muscle shortening is determined by the movement of actin filaments over myosin ones. A convenient and adequate model for studying this movement in general and its characteristic velocities in particular is *in vitro* motility assay, which allows the actin-myosin interaction characteristics to be determined at the level of isolated proteins. A fluorescently labeled actin or a regulated thin filament in the presence of ATP moves on the surface of a flow cell covered with rat myosin isolated from the same hearts from which the trabeculae and papillary muscles were excised. Regulated thin filament, unlike bare F-actin, includes actin, tropomyosin and troponin, can interact with calcium ions in solution. Troponin C can bind with calcium in the solution of the flow cell; as a result of this interaction, tropomyosin shifts, revealing myosin binding sites on actin globules. We can judge about actin-myosin mechanical interaction by the characteristics of how the filaments move and stop. Changes in these characteristics depending on calcium concentration in the solution in the case of a regulated thin filament enable one to study the calcium regulation mechanisms of actin-myosin interaction (Gordon et al., 1997). Actin was obtained from rabbit skeletal muscle according to standard procedure (Pardee and Spudich, 1982). Cardiac troponin was isolated from the left ventricle of

a bovine heart as described (Potter, 1982). Recombinant tropomyosin was obtained as previously described (Matyushenko et al., 2014). The use of contractile and regulatory proteins extracted from different species and combined in a motility assay is a common practice (Gordon et al., 1997; Alpert et al., 2002; Shaffer et al., 2010).

Thin filaments were constructed from actin, troponin, and tropomyosin by mixing these proteins in the following concentrations: 400 nM rhodamine-phalloidine labeled F-actin, 100 nM troponin and 100 nM tropomyosin at 4 °C in AB buffer. Protein ratio in the thin filaments was checked by 10% SDS-PAAG (Laemmli, 1970).

An *in vitro* motility assay was performed as described previously (Nikitina et al., 2008a). Briefly, a 50-μl flow cell was constructed with a nitrocellulose coating. First 50 μl of myosin at a concentration of 300 μg/ml in AB buffer (25 mM KCl, 25 mM imidazole, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM DTT, pH 7.5) containing 0.5 M KCl was loaded in the flow cell. After 2 min the flow cell was rinsed first with a high and then with a low ionic strength AB buffer to remove unbound myosin. Then 50 μl of 0.5 mg/ml bovine serum albumin (BSA) in AB buffer was added for 60 s to block any exposed nitrocellulose surface. Further 50 μl/ml of non-labeled F-actin in AB buffer with 2 mM ATP was added and incubated for 5 min to block nonfunctional myosin heads. The flow cell was rinsed three times with AB buffer. Then 50 μl of 10 nM rhodamine-phalloidine labeled thin filaments in AB buffer were added for 5 min. AB buffer contained 100 nM troponin and 100 nM tropomyosin to prevent dissociation of the thin filaments. Unbound thin filaments were washed out with AB buffer. Finally the cell was washed with AB buffer containing 0.5 mg/ml BSA, 3.5 mg/ml glucose, 0.02 mg/ml catalase, 0.15 mg/ml glucose oxidase, 20 mM DTT, 2 mM ATP and 0.5% methylcellulose, 100 nM troponin, 100 nM tropomyosin and free calcium. The required concentration of free calcium was obtained by appropriate Ca–EGTA ratio (Nikitina et al., 2008a).

Fluorescently labeled thin filaments were visualized by an Axiovert 200 inverted epifluorescence microscope equipped with a 100×/1.45 Oil alpha Plan-Fluar objective (Carl Zeiss) and an EMCCD iXon-897BV camera (Andor Technology). Typically 10 fields of 30 s each were recorded in every flow cell. Data were analyzed using GMimPro software (Mashanov and Molloy, 2007). Typically, the velocities of > 100 individual filaments were averaged to determine the mean values ± SD.

3. Results and discussion

3.1. Main evidence of lead toxicity on the organ-systemic level

The quantitative values of all indices for the organism's status listed in Section 2.1 were presented and discussed in detail in our recent study (Protsenko et al., 2018), so the reader is referred to this paper for detailed information. Briefly, the lead-treated group (Pb) had a substantial number of these indices differing from the control group, pointing in total to the development of subchronic intoxication. Along with signs of the latter commonly observed under the impact of any toxic agent (e.g. delayed weight gain), we revealed ones which were more or less specific to the effects of lead. These were, first of all, a manifold increase in the concentration of δ–aminolevulinic acid and coproporphyrins in the urine, a reduction in the hemoglobin content of the blood as well as in both RBC count (along with a reduced average RBC volume) and hematocrit, paralleled by a sharp increase in the proportion of reticulocytes. Less specific to lead toxicity effects but observed by other researchers as well (e.g., Noori Mugahi et al., 2003; Farkhondeh et al., 2014) was marked neutrophylic leukocytosis. The absolute number of lymphocytes in blood was also significantly increased. We are not aware of any literature mentioning this effect as caused by lead intoxication. Finally, we found an increased thrombocyte count and thrombocrit, which have also been described by some authors as hematotoxic effects of lead (Noori Mugahi et al., 2003; Al Momen, 2010).

Lead is widely known for its renal toxicity (e.g. Nolan and Shaikh, 1992; Kireyeva et al., 2010; Katsnelson et al., 2012; Varaksin et al., 2014; Panov et al., 2015; Minigalieva et al., 2017), which in this experiment manifested itself in an increase in kidney mass. Hepatotoxicity is characteristic of virtually all heavy metals, and in our experiment it was not only increased liver mass that was indicative of it but also such markers as reduced total protein, albumin and globulin contents of the blood serum (the A/G index being unchanged), and a sharp increase in the level of aspartate aminotransferase in the latter (with de Ritis coefficient doubled). The well-known neurotoxicity of lead (Rius et al., 1986; Bressler and Goldstein, 1991; Adonaylo and Oteiza, 1999; Ma et al., 1999; Nehru and Sidhu, 2001; NourEddine et al., 2005; Deveci, 2006; Sanders et al., 2009; Mason et al., 2014) might explain an increase in brain mass and a slight weakening of the exploratory behavior in the context of markedly and statistically significantly reduced general motor activity.

In both lead-treated groups in our experiment we observed a notable, although statistically non-significant, increase in total cholesterol and a significant reduction in high-density lipoprotein cholesterol (with an actually unchanged level of low-density lipoprotein cholesterol) and a statistically significant (nearly two-fold) increase in triglyceride level. Thus, on the whole, what we dealt with was atherogenic changes in the blood lipid profile. Although Allouche et al. (2011) did not observe such changes, in the literature one finds other data on the effects of lead on blood lipids that are in agreement with ours (Tarugi et al., 1982; Skoczyńska et al., 1993).

The majority of the above-described shifts were observed only in the two lead-treated groups (Pb and Pb + Ca). However, where there was a statistically significant quantitative difference between the effects of lead when given calcium and without it, the sign of the difference pointed to attenuation of the toxic effect, which is consistent with the numerous published data on calcium-lead antagonism, including our own (Barton et al., 1978; Rius et al., 1986; Bogden et al., 1992; Varnai et al., 2001; Privalova, 2005; Katsnelson et al., 2007, 2008; 2009; Savchenko et al., 2015).

3.2. Myocardial contractility in isotonic and physiological modes

The amplitude and velocity of muscle shortening both depend on the level of afterload, which is reflected by the curve for the dependence of maximal shortening velocity on afterload applied (force-velocity relationship). The value of the maximal shortening velocity in the absence of afterload correlates with myosin ATPase activity (Cappelli et al., 1989). Since absolute shortening velocity of the muscle depends on its length, the maximal shortening velocity was normalized to this length (V_{MAX}/L_W, where L_W is the working length of the muscle and is equal to 95% L_{MAX} in our case). The afterload values were normalized to peak isometric contraction (P/P_0). The force-velocity curves for the ventricular trabeculae and papillary muscles of rats in all experimental groups have typical shape revealed on any level of myocardial tissue – from molecular to multicellular (Sonnenblick, 1962; de Tombe & ter Keurs, 1990; McDonald and Herron, 2002; Nikitina et al., 2008b) and are shown in Fig. 2.

No statistically significant differences in force-velocity relationship were observed for the ventricular trabeculae of rats from all experimental groups (Fig. 2A). In contrast, significant differences were revealed in the papillary muscle preparations between Pb group and control group (reduced velocity in the Pb group over the entire range of afterloads). Also, we found significantly lower values of peak shortening velocity (virtually for the whole range of afterloads) in the papillary muscles of both lead-treated groups (Pb and Pb + Ca) if compared to the corresponding groups for trabecular preparations ($P < 0.05$). Surprisingly, untreated groups (either control or Ca) showed no differences between papillary muscles and trabeculae in terms of force-velocity relation.

Extrapolation of the curves to the intersection with the Y axis provides an estimate of the shortening velocity under zero load which is known to be correlated with myosin ATPase activity: the higher the

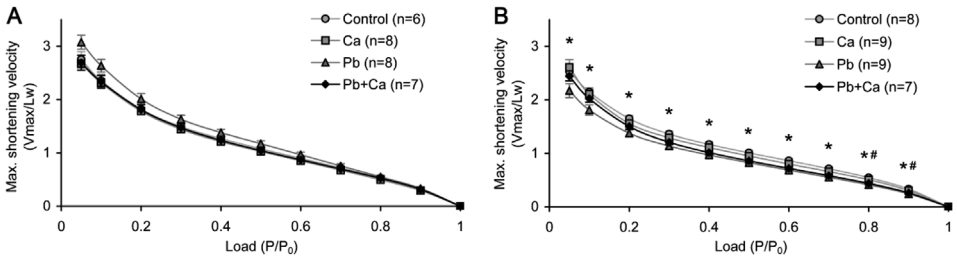

Fig. 2. The relationships between the maximal velocity of isotonic shortening normalized to muscle length (V_{MAX}/L_W, L_W – working muscle length) and the afterload (P/P_0) obtained for right ventricular trabeculae (A) and papillary muscles (B) of rats in all experimental groups. The diastolic (working) length of the muscles corresponds to 95% L_{MAX}. Stimulation frequency 1 Hz, temperature 30°C. * – differences between the control and Pb groups are significant for $P < 0.05$, # – differences between the Ca and Pb + Ca groups are significant for $P < 0.05$ (Mann-Whitney U test).

ATPase activity of the myosin, the higher the rate of unloaded shortening (Cappelli et al., 1989). The maximal velocity of papillary muscle shortening was as follows (units are V_{MAX}/L_W): control group = 3.05 ± 0.15, Pb group = 2.69 ± 0.19, Ca group = 3.36 ± 0.23, Pb + Ca group = 3.03 ± 0.13.

Admittedly, such indirect estimate of unloaded shortening velocity is inaccurate. We therefore performed experiments on myosin isolated from the ventricles of the same hearts in control and Pb groups. Using an *in vitro* motility assay, we investigated the rate of unloaded movement of thin filament at a calcium concentration of the solution of 10^{-6} M along myosin in these two groups. The sliding velocity on myosin was 2.0 ± 0.25 μm/s (n = 8) for the control group and 1.5 ± 0.2 μm/s (n = 8) for the lead-treated group, i.e. the rate of unloaded movement in the Pb group dropped by 25% compared with the control. The above confirms qualitatively the indirect estimates of the differences in the unloaded shortening rates obtained on papillary muscles, quantitatively even exceeding these differences (25% decrease in the *in vitro* motility assays and 12% in papillary muscles). These differences in movement rates are directly related to the shift in the cardiac myosin isoform ratio under lead intoxication towards V3 isomyosins which are slower in velocity and lower in ATPase activity than V1. This shift was established on the basis of the same body of experimental data and reported by us previously (Protsenko et al., 2018). These data may indicate a possible onset of moderate myocardial hypertrophy in the Pb group. Because the entire right ventricle was used for performing gel electrophoresis and *in vitro* motility assays, any possible regional distinctions in isomyosin contents and in their velocity characteristics were not identifiable. At the same time, our data obtained on muscle preparations provide evidence of regional non-uniformity: changes in the force-velocity characteristics of the preparations from the Pb group compared with the control were weakly expressed for the trabeculae and much more pronounced for the papillary muscles. We reported recently that a well-characterized subchronic lead intoxication also induced changes in the *isometric* force generated by the ventricular myocardium while shifting the isomyosin ratio towards the slow isoform, which may indicate myocardial hypertrophy (Protsenko et al., 2018).

An important aspect of the study was evaluation of the effect exerted by subchronic lead intoxication on the work produced by isolated ventricular trabeculae and papillary muscles of the rats of the four experimental groups. The work produced by the muscles was calculated by the area of the loops formed by curves representing changes in active mechanical tension and muscle length in the course of physiological mode of contraction in the in each group of rats with subsequent averaging over the entire range of afterloads. As an example, we show the tension-length loops for trabeculae and papillary muscles under afterload of 0.5 P_0 (Fig. 3).

The amount of work produced by the trabecula and papillary muscle preparations as a function of afterload is shown in Fig. 4 (A and B). An extremum in this dependence can be seen at the very middle range of afterloads (0.4–0.6 P_0) in the preparations from all groups of rats with no any significant differences between the groups. It should be noted that the amount of work performed by the papillary muscles of a group is tended to be higher compared to the trabeculae in the same group (compare Fig. 4A and B) while the significant differences were found for individual afterload values, not for the whole range of afterloads.

It is notable that the amount of work (i.e. the areas of the loops formed by the phase trajectories in Fig. 3) produced under lead intoxication in the trabecular preparations is due to an increase in the shortening amplitude with a drop in the tension compared with the controls. An opposite tendency is observed in the papillary muscle preparations (compare Fig. 3A and B). Thus, both types of heart muscle have demonstrated adaptation to lead intoxication in terms of ability to produce mechanical work, which in the whole organism is of greatest hemodynamic importance.

To estimate the effect of lead on calcium regulation of myocardial relaxation, we used the index of load dependent relaxation (*ILDR*) as the ratio of $(t_i - t_a)/t_i$ (Fig. 5) where t_a is the duration of the isotonic phase of a twitch under a given afterload, and t_i is the duration of the part of the isometric twitch where the force remains higher than this afterload. This characteristic determines the mechanical dependence of calcium activation of cardiomyocyte contraction-relaxation cycles (Lecarpentier et al., 1987; Housmans and Murat, 1988; Dobrunz and Berman, 1994; Katsnelson et al., 2000).

In the isotonic loading mode, *ILDR* characterizes the effect of the magnitude of afterload on the duration of the isotonic phase in the myocardial contraction-relaxation cycle. This effect is controlled by the impact of dynamic changes in cardiomyocyte length on calcium activation of contraction. The closer the *ILDR* to 0, the less load-dependent is the isotonic contraction-relaxation cycle. If *ILDR* = 0 for all afterloads, there is no load dependence at all, and the durations of the isotonic cycle and isometric cycle in this case are the same. For normal heart muscle, the characteristic values of *ILDR* are higher than 0 for afterloads lower than 0.5 P_0, and the lower the afterload, the higher the value of *ILDR*. For large afterloads the *ILDR* can become negative which may mean overactivation of myofilaments under minor shortening and high load.

The typical records of isometric and isotonic twitches obtained for control and Pb groups of muscles and used to calculate *ILDR* values are shown in Fig. 6. Estimation of *ILDR* by a series of isotonic contractions of isolated trabecular preparations revealed a lack of any effect of lead (Pb) and lead plus calcium-containing bioprotector (Pb + Ca) on the dependence of this index on afterload compared with the myocardium of the control animals (Fig. 7A and B). In particular, an increase in the duration of trabecular isometric contractions in the Pb group was highly proportional to the increase in the duration of the isotonic contraction plateau phase in this group (see Fig. 6). This ensured that the *ILDR* values measured under any afterload in all trabecular preparations were quantitatively similar in all studied groups of rats. For 0.5 P_0, the average value of *ILDR* amounted to 0.186 ± 0.006 for control group, 0.165 ± 0.009 for Ca group, 0.173 ± 0.005 for Pb group, and 0.177 ± 0.010 for Pb + Ca group.

In contrast to trabeculae (Fig. 7A), the papillary muscle preparations from lead-treated rats displayed a statistically significant increase in *ILDR* compared with the control group in the wide range of afterloads (Fig. 7B). Thus, for instance, for 0.5 P_0 the average value of *ILDR* was

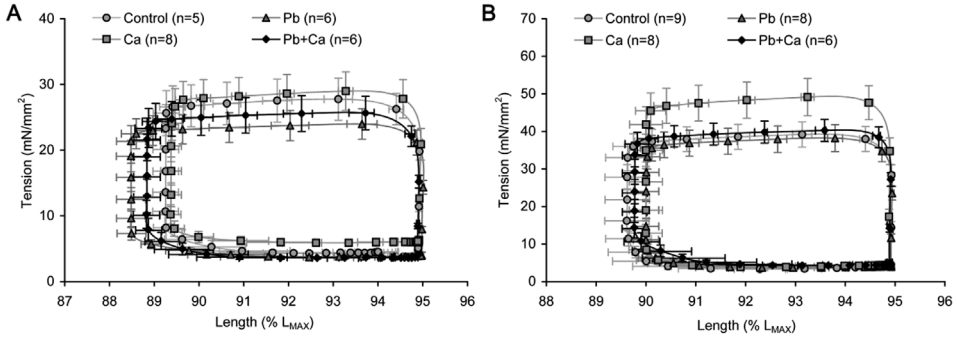

Fig. 3. Superposition of mechanical tension—length loops obtained under a physiological mode of contraction set for trabecular preparations (A) and papillary muscle preparations (B) of rat right ventricles from all groups studied. Tension is expressed in absolute values (mN/mm^2), and changes in muscle length as % of L_{MAX}. The diastolic (working) length of the muscles corresponds to 95% L_{MAX}. Stimulation frequency 1 Hz, temperature 30^{O}C.

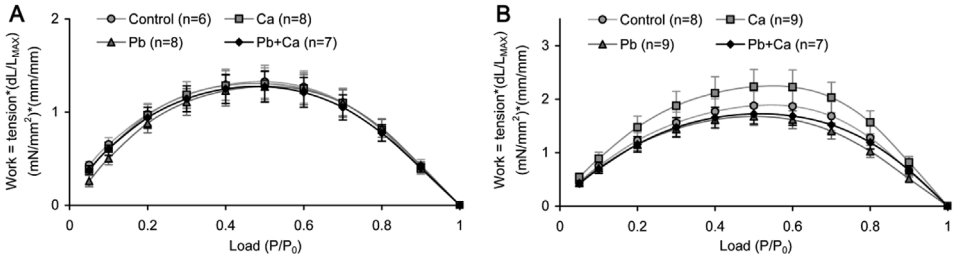

Fig. 4. The relationships between work and afterload (P/P_0) obtained for trabeculae (A) and papillary muscles (B) of rat right ventricles from all groups studied. Work is expressed through units of muscle mechanical tension and relative length as a fraction of L_{MAX}, i.e. normalized work unit – (mN/mm^2)*(mm/mm). The diastolic (working) length of the muscles corresponds to 95% L_{MAX}. Stimulation frequency 1 Hz, temperature 30^{O}C.

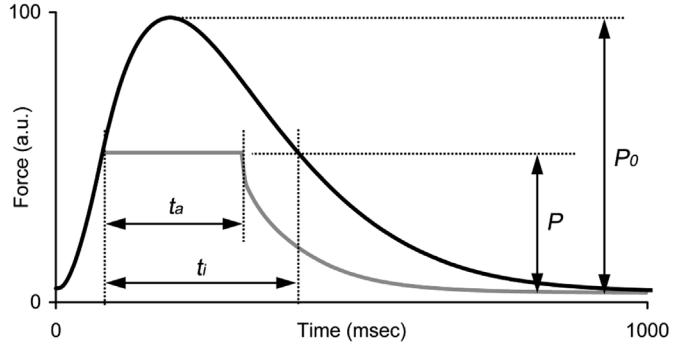

Fig. 5. The diagram for determination of the index of load dependent relaxation. P_0 – peak isometric force, P – afterload (set as a fraction of P_0), t_a – the duration of force plateau, t_i – the duration of the part of isometric twitch for the forces above or equal to the P value. See the text for explanations.

0.179 ± 0.008 in control group and 0.225 ± 0.012 in Pb-group. As similar to force-velocity relationship, the significantly different mean values of *ILDR* were found for papillary muscles *vs* trabeculae in Pb group (in the range of 0.05–0.9 P_0) and in Pb + Ca group (0.1–0.6 P_0) but not in both groups not treated by lead (Control and Ca).

Increased *ILDR* points to a weaker load dependence of the papillary muscles in rats from the Pb group compared with the control. The mechanisms responsible for the load dependence of isotonic cycles and factors influencing the weakening of load-dependence up to its complete disappearance have been discussed in many studies (Lecarpentier et al., 1987; Housmans and Murat, 1988; Dobrunz and Berman, 1994; Katsnelson and Markhasin, 1996; Katsnelson et al., 2000). Without going into details, we should emphasize that the effect of load dependence is generally determined by the existence of mechano-calcium feedback in cardiomyocytes: changes in the mechanical conditions of contraction (load) and in its mechanical characteristics (length, shortening velocity) modify significantly the calcium activation of the contraction cycle. This mechano-calcium feedback is the principal mechanism enabling the muscle to adapt to dynamically changing conditions of the contraction cycle. Thus, the reduced load dependence of *ILDR* testifies to the attenuation of this feedback and, hence, to partial loss of adaptability by papillary muscle under lead intoxication.

4. Conclusion

Let us summarize the findings of the experiments carried out on

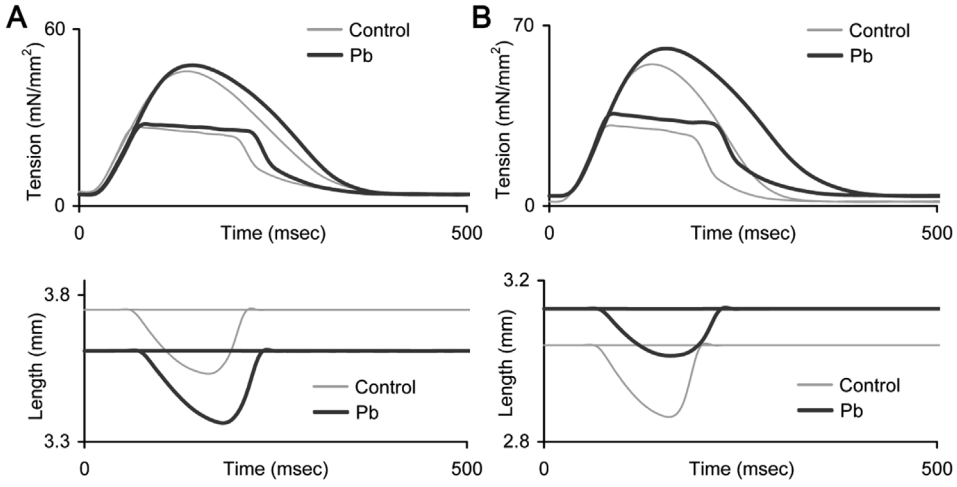

Fig. 6. A representative case of isometric and afterload contractions under afterload of 0.5 P_0 for right ventricular trabeculae (A) and papillary muscles (B) of rats from the control group (thin light-grey lines) and after subchronic intoxication with lead (thick dark-grey lines). The contractile response is given in absolute units (mN/mm^2 for tension, mm for length). The diastolic (working) length of the muscles corresponds to 95% L_{MAX}. Stimulation frequency 1 Hz, temperature 30^{O}C.

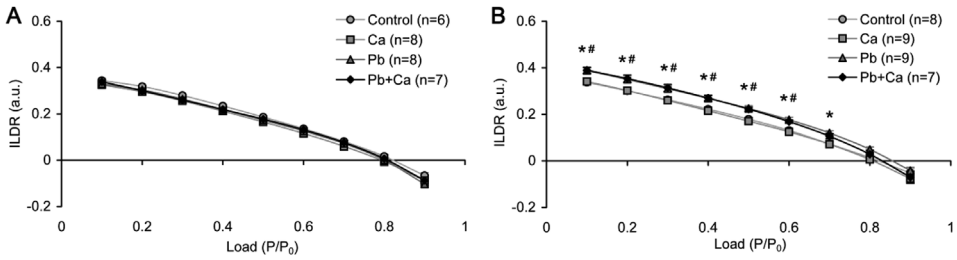

Fig. 7. The relations of the index of load-dependent relaxation (*ILDR*, see the text for explanations) on afterload (P/P_0) obtained for the ventricular trabeculae (A) and papillary muscles (B) of rats from all experimental groups. The diastolic (working) muscle length is 95% L_{MAX}. Stimulation frequency 1 Hz, temperature 30°C. * – differences between the control and Pb groups are significant at $P < 0.05$, # – differences between the Ca and Pb + Ca groups are significant at $P < 0.05$ (Mann-Whitney *U* test).

tissue and molecular levels presented both in this paper and in the previous publication (Protsenko et al., 2018). Using isolated preparations of trabeculae and papillary muscles as well as myosin from the right ventricles of rats exposed subchronically to lead by intraperitoneal injections, we have shown the following:

- changes in most of the basic characteristics of isometric and afterload contraction-relaxation cycles are more pronounced for papillary muscles as compared with trabeculae, and this difference, demonstrated by us for the first time, needs further elaboration and requires additional investigations;
- the amplitude of isometric contraction force is stabilized by an increase in the time to peak force, particularly at short lengths, as well as by a decrease in the rate of isometric contraction force development;
- maximal rates of isotonic shortening for all afterloads in the papillary muscle are lower in the lead-exposed group compared with the control and the other groups, which is consistent with the data obtained by us in an *in vitro* motility assay, where the maximum velocity of unloaded movement of a regulated filament along myosin was considerably lower for the protein of the lead-exposed group in comparison with the control group;
- a reduction in the velocity of both the thin filament and the velocities of isotonicshortenings correlates well with a shift in the expression of isomyosins towards slower V3 isomyosin under subchronic lead intoxication of rats revealed by means of gel electrophoresis; V1 and V3 rat isomyosins develop an equal isometric force (Malmqvist et al., 2004), which explains the persistence of the peak force of isometric contractions in the lead-exposed group compared with the control;
- a drop in the passive (diastolic) mechanical tension takes place;
- no changes in the efficiency of the contractile apparatus estimated by the work produced by muscles over the entire range of afterloads were discovered in both types of muscle, which provides evidence of myocardial adaptation to the above-mentioned adverse effects of subchronic lead intoxication;
- at the same time, an increase in the *index of load dependence* in the papillary muscle preparations from rats exposed to lead (with or without supplementary calcium) points to a reduced ability of these muscles to adapt to dynamic conditions of mechanical loading;
- a calcium-containing supplement has been shown to produce a moderate protective effect on both organism-level lead intoxication and related changes in rat myocardium, which are manifest in both isometric and isotonic modes of muscle contraction.

The above summary suggests an impaired contractility of the cardiac muscle due to the subchronic lead intoxication. This impairment may be partly explained by the remodelling of the myocardium on molecular level, in particular by a change in the isoenzyme structure of the myosin and a corresponding reduction in the velocity of actin sliding over myosin. Admittedly, the objects of intracellular remodelling in this case could also be structures which participate in calcium handling and are responsible for the regulation of the contractile act. We have therefore made plans to conduct further studies on cell preparations to explore the mechanical activity and calcium transient of isolated cardiomyocytes.

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