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Metabolic Features of Heart Failure with Different Etiology

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Abstract: Dilated Cardiomyopathy (DCM) and Chronical Degenerative Valvular Disease (CDVD) are very common in dogs nowadays. However, due to modern level in oncology we can meet chemotherapy-induced cardiomyopathies more often. We have lack of information about pathogenic mechanisms underlying Doxorubicin-induced Cardiomyopathy (DoxCM) and features differentiating it from CDVD and DCM. In this study we investigate several metabolic features of doxorubicin-induced cardiomyopathy, DCM-like phenotype cardiomyopathies and CDVD. We observed some stages of myocardium glucose metabolism in order to estimate life potential of end stage myocardium and respectively survival of patient. The study population consisted of 46 dogs of different age, sex and breed. These dogs were subgrouped due to diagnosis: Healthy dogs without heart failure, DCM, CDVD and DoxCM. Fresh myocardial biopsies taken immediately after euthanasia from left ventricular apex, freezed in liquid nitrogen for ELISA and biochemical study. Differences in metabolic profile found between of CDVD and healthy dogs were not significant. At the same time doxorubicin induced cardiomyopathy and DCM-like phenotype myocardial disease had similar changes. It should be mentioned, DCM patients had previously long history of treatment, but DoxCM group-not. Differences between DoxCM, DCM and CDVD were significant and can be explained by changes what suffering myocardium undergoes. Rapid or slow developing energy depletion leads to myocardiocytes death and heart failure. Doxorubicin-induced cardiomyopathy is far more dangerous due to rapidness of development in observed study. Myocardium, due to lack of time, had no adapting ability for acute energy depletion and massive cell death. This preliminary study shows changes induced by doxorubicin (Dox) in dogs. These findings mostly connected with mitochondrial disturbances, insulin resistance and energy depletion. In this study also shown several features connected with development of CDVD and differences from DCM.

Keywords: Myocardial Metabolism, Cardiac Histology, Cardiac Ultrastructure, Doxorubicin-Induced Cardiomyopathy

Introduction

Heart failure, despite its etiology, is coupled with significant disturbances of cardiomyocytes energy supply. Thank to Hermann and Dechred (1939) we keep in mind that heart failure is a sequence of myocytes energy starvation.

Myocardium is one of the most energy-depended structure, it demands about 6 kg of ATP a day. In order to sustain efficient energy supply it has advanced system producing enough ATP. First step is consuming of glucose and Free Fatty Acids (FFA) from blood, then glycolysis and beta-oxidation provide intermediates for Tricarboxylic Acid Cycle (TCA). After that metabolites driven to mitochondrial oxidative chain, which phosphorylate ADP to ATP. In the end, Creatine Kinase (CK) system organize transport of macroergic phosphates from mitochondria to contractile sites. Phosphocreatine (PCr) is the most important substrate to support energy demands of myocardium (Ingwall and Weiss, 2004; Ingwall, 2002; 2009; Lopaschuk *et al*., 2002; Paolisso *et al*., 1994; Ventura-Clapier *et al*., 2004).

Oxidative phosphorylation-is the main source of macroergic phosphates. ATP synthesis is strongly connected with formation of Reactive Oxygen Species (ROS), what usually utilized by cell antioxidant system.

Disturbances in mitochondrial oxidation leads to heart failure, due to decreased ATP production for myofibrils. Following changes are associated with structural damage of mitochondria, lost of its membrane potential, in the end we have decreased affinity to phosphorylation recipients (ADP, AMP, creatine) (Ingwall and Weiss, 2004; Ingwall, 2002; 2009; Lopaschuk *et al*., 2002; Ventura-Clapier *et al*., 2004).

Contractility suppress leads to activation of catecholamine supportive system. Activation of beta-adrenergic receptors increases heart rate and ATP consumption, this situation ends with high amounts of ADP and negative inotropic effect (Liu *et al*., 2001; Neubauer, 2007).

There are lots of therapeutic agents with cardiotoxic effect. The significant amount of these drugs are used in oncology, due to its negative effect on neoplasia cells. Unfortunately, it is very difficult to organize target action of chemotherapy, so toxicity is mostly have effect on whole organism. Due to ROS-producing effect of chemotherapy agents, genetic apparatus of active dividing cells is damaged in both neoplasia cells and normally proliferating blood cells. Also high amounts of produced ROS are toxic to cells which highly depend on oxidative phosphorylation. Cardiomyocytes are one of them, it need to be mentioned, their vulnerability to chemotherapy drugs is also connected with very low cell antioxidant system activity (Takemura and Fujiwara, 2007; Yeh *et al*., 2004; Zucchi and Danesi, 2003).

As in human medicine, anthracyclines are most common chemotherapy drugs. Doxorubicin is leading agent due to its wide action specter. Unfortunately, chemotherapy always coupled by toxic side effects, which has significant influence on therapy quality and can a cause change of standard scheme of treatment (Aries *et al*., 2004; Lebrecht *et al*., 2005; Minotti *et al*., 2004; Takemura and Fujiwara, 2007; Yeh *et al*., 2004).

Negative effects of anthracyclines have influence on many organs and systems, significant alterations are usually connected with gastro-intestinal, hemopoetic and cardiovascular systems. Due to the fact that most oncology patients are geriatric, predispose to pay more attention to myocardium condition (Klimas, 2012; Takemura and Fujiwara, 2007).

Standard echocardiography and electrocardiography sometimes is not sufficient to find developing disease, due to inability to define state of individual cardiomyocytes, which could become sites of fibrosis and ectopic electro activity. Doxorubicin toxic effects are less common in dogs than in human, due to differences in chemotherapy scheme. In human medicine DoxCM often develop in several decades after treatment and in case of overcoming cumulative threshold of 450-500 mg\m^2 (Swain *et al*., 2003). In veterinary medicine threshold dose is about 160-200 mg\m^2 (Mauldin *et al*., 1992).

In practice, we have two types of cardiotoxicity: Acute-connected with myocardial electroactivity disturbances and arrhythmias, which have to be medicated; and chronic toxicity-leading to cardiomyocytes death, fibrosis, systolic and diastolic dysfunction and metabolic disturbances in rest of myocardial cells (Swain *et al*., 2003; Takemura and Fujiwara, 2007; Von Hoff *et al*., 1979).

The leading pathogenesis mechanism of doxorubicin toxicity is still unknown, but already have been defined key pathological effects of anthracycline: Synthesis of reactive oxygen species (mitochondrial, No-connected, NAD(P)H-depended); DNA intercalation; complex bound doxorubicin-iron; alteration of Ca ions transport is sarcoplasmic reticulum; decreasing of macroergic phosphates synthesis; endothelin dysfunction; myocardial remodeling; oxidative stress (Bachur *et al*., 1977; Doroshow, 1983; Fogli *et al*., 2004; Gewirtz, 1999; Hasinoff, 1998; Kelvin and James, 1986; Myers *et al*., 1977; Nohl *et al*., 2003; Outomuro *et al*., 2007; Tokarska-Schlattner *et al*., 2006; Weinstein *et al*., 2000; Zhou *et al*., 2001b; 2001a; Zuppinger *et al*., 2007).

Compensatory mechanisms in developing cardiomyopathy include: Increasing in FFA consumption; adrenergic system activation; transcardial hemodynamics modulation; parenchymal and stromal remodeling.

From the first steps of heart failure, increased FFA consumption is coupled with catecholamine activation, until development of decompensation, which connected with metabolic turn to glycolysis (Ashrafian *et al*., 2007; Eisenhofer *et al*., 1996; Kostis and Sanders, 2005; Lopaschuk *et al*., 2010; Opie *et al*., 1979; Opie, 2006; Paolisso *et al*., 1991; Zucker, 2006).

FFA transport to cardiomyocytes is implemented by several ways: Passive diffusion and specific transport proteins. After diffusion of long-chain fatty acids in myocyte, they metabolized into acyl-Coenzyme A (acyl-CoA) and transported to specific protein on mitochondrial membrane for acyl-CoA synthase interaction. Active transport is supported by FATP1, FATP6, CD36, next step for this way obtained long-chain fatty acids is bounding with CoA and metabolizing in beta-oxidation. Some of these FFA are esterificated and stored as lipid drops in cytoplasm (Kampf *et al*., 2007; Luiken *et al*., 2002; 1999; 1997; van der Vusse *et al*., 2000).

Myocardial FFA metabolism is also influenced by secondary messenger AMP-Activated Protein Kinase (AMPK), which regulated by AMP\ATP ratio. There are several sites of this molecule action: Inhibition of malonyl-CoA, suppressing acyl-CoA synthase and decreasing of FFA storage; decreased malonyl-CoA prevent carnitine palmitoyltransferase inhibition and transport of FFA to mitochondria; stimulate expression of FATP and CD36 on outer cardiomyocyte membrane (Ardehali *et al*., 2012;

Lionetti *et al*., 2011; Sack *et al*., 1996; Shibayama *et al*., 2015; Tokarska-Schlattner *et al*., 2005).

In addition, AMPK influences of glycolytic pathways of energy production: Increasing glucose transport due to activation of GLUT 1 and GLUT 4; inhibiting of glycogen synthesis by deactivation of glycogen-synthase (Anwan and Saggerson, 1993; Bonen *et al*., 1999; Fueger *et al*., 2007; Long *et al*., 2005; Luiken *et al*., 2003; Saha *et al*., 2000; Shearer *et al*., 2005; Steinberg and Kemp, 2009; Stoppani *et al*., 2002).

Family of glucose transporters in myocardium is mostly represented by GLUT 1, GLUT 4, GLUT 12. GLUT1 are insulin-independed and connected with base glucose supplementation. GLUT 12 is weakly expressed on cardiomyocyte outer membrane, but its role still unclear (Becker *et al*., 2001; Bell *et al*., 1993; Fischer *et al*., 1997; Stanley *et al*., 1997; Wheeler *et al*., 1994).

Nowadays role of GLUT4 is most significant in metabolic aspects of cardiomyopathies and local Insulin Resistance (IR) development. This type of transporter implements glucose supplementation to cytoplasm organelles, especially for T-tubules, due to group of associated Ca+2 ion pumps which demand high amounts of ATP, got mostly from glycolysis (Fazakerley *et al*., 2009; Lauritzen *et al*., 2006; 2008; Wang *et al*., 1996).

Important step in understanding of metabolic part of heart failure is development of local IR. This state is based on persistent hyperinsulinemia. Adrenergic reaction coupled with heart failure leads to activation of glycomobilization system and Ins co-activation and, in parallel, catheholamine-induced lipomobilization (noradrenalin). Ins stimulate exocytosis of GLUT4 and CD36, which helps to get sufficient amounts of energy substrates for oxidative phosphorylation and glycolysis. But due to difference in Ins receptors we can get IR. This effect based on affinity of receptor to Ins, during chronic hyperinsulinemia receptors with low affinity suppress more active and worsen IR (Cook *et al*., 2010; Davila-Roman *et al*., 2002; De Meyts *et al*., 1976; 1973; Lionetti *et al*., 2007; Nikolaidis *et al*., 2004; Shanik *et al*., 2008; Taylor *et al*., 2001; Witteles *et al*., 2004).

In parallel, Ins-stimulated FFA consumption leads to their storage in cytoplasm, formation of lipid drops, increasing of acyl-CoA- FFA bounds concentration. These metabolites inhibit Pyruvate Dehydrogenase (PDH) activity and suppress glycolysis and exceed oxidative potential of mitochondria. Due to PDH inhibition glycolysis intermediate compounds are redirected to glycogen genesis, what brings accommodation of glycogen, inability to use it energy supplement, electroactivity disturbances because of storage disease. Also metabolites could act as a stimulators of hexosamine biosynthesis. Fructose-6-phosphate is metabolized to uridinediphosphate-N-acetylglucosamine, what stimulates O-associate glycolisation of contractile filaments, Ca ion pumps of sarcoplasmic reticulum. In the end it leads to apoptosis (Ashrafian *et al*., 2007; Clark *et al*., 2003; Hu *et al*., 2005; Kotoulas *et al*., 2004; Lopaschuk *et al*., 2010; Nikolaidis *et al*., 2001; Randle *et al*., 1963; Rechelt *et al*., 2013; Xu *et al*., 2010).

Creatine kinase complex, which implement distribution of phosphates between ATP and creatine, also damages in pathological myocardium. Decreased activity of this enzyme suppress PCr\ATP ratio, what leads to low ability to transport phosphates to ADP (Lopaschuk *et al*., 2010; Nahrendorf *et al*., 2005; Neubauer *et al*., 1997; Oleynikov *et al*., 2015; Randle *et al*., 1963; Wallis *et al*., 2005).

Lactate-Lactate Dehydrogenase (LDH)-pyruvate system helps to obtain additive amounts of pyruvate in order to retain energy substrates or to withdraw exceed amounts of pyruvate, metabolizing it to lactate, obtained due to inhibition of PDH by Randle cycle reactions. In heart failure, high adrenergic stimulation and compensatory mechanisms increase utilization of FFA what leads to inhibition of PDH and idle pyruvate metabolized by LDH to lactate and shuttles it in circulating blood. This process decreases general amounts of pyruvate and lactate in cytosol and rise in lactate\pyruvate ratio, negatively alternating energy supplementation in submembrane ion pumps (Des Rosiers *et al*., 2011; Lopaschuk *et al*., 2010; Oleynikov *et al*., 2015; Randle *et al*., 1963; Shibayama *et al*., 2015).

Additive component of energy deficit is electrolyte disturbances in cardiomyocyte metabolism. Under adrenergic stimulation there is active calcium transport and, in parallel, sodium retention in cardiomyocyte. High amount of Na suppress activity if Ca+2\Na+ pump and secondary cardiomyocyte has significant Ca+2 ions in cytoplasm. Also high Na concentration leads to membrane depolarization disturbances and myocyte edema (Pieske *et al*., 2002).

On the first stage increased intracellular Ca+2 flux stimulates Ca+2-induced Ca+2 release. This mechanism helps to increase contractile ability in stress situations. In case of chronic stimulation, there is energy depletion of tubular system Ca+2 ionic pump, due to its high dependence to glycolytic ATP production. It could be explained by the fact of PDH complex inhibition, described earlier. Absence of effective Ca+2 elimination leads to diastolic dysfunction and depolarization alteration (Avero *et al*., 1995; Dodd *et al*., 1993; Fabiato, 1983; Feher and Lipford, 1985; Jenden and Fairhurst, 1969; Lewartowski *et al*., 1990; Meissner and Henderson, 1987; Northover, 1991; Sutko and Airey, 1996).

In common, metabolic aspects of heart failure are described in many works and the common view on metabolic side of cardiomyopathy pathogenesis is already exist. However, differences between primary and secondary cardiomyopathies are not defined (Neubauer, 2007).

Further we need to elucidate myocardial dysfunctions appeared after doxorubicin treatment. In common anthracyclines can cause myofilaments damage, suppression of their resynthesis; energy metabolism alteration, following depletion of PCr\ATP ratio, AMPK inhibition; damage of lipid structures of mitochondria by ROS; Ca+2 storage in mitochondrial matrix, their petrification (Hrelia *et al*., 2002; Ingwall, 2004; Minotti *et al*., 2004; Olson and Mushlin, 1990; Weinstein *et al*., 2000; Zou *et al*., 2003).

Acute effects of Dox are based on rapid Ca+2 flux from sarcoplasmic reticulum, activation of Ca+2-dependened Ca+2 channels and inhibition of concurrent attachment of Mg+2 ions to Ca+2 receptors. Dox-induced increasing of Ca+2 in cytoplasm leads to Na retention, suppression of Na\K pump, cardiomyocyte edema and high risk of arrhythmogenicelectroactivity. Sometimes, Dox-induced alteration can cause damage of sarcoplasmic reticulum (Doroshow, 1991; Holmberg and Williams, 1990; Minotti *et al*., 2004; Nagasaki and Fleischer, 1989; Pessah *et al*., 1992; Zucchi and Ronca-Testoni, 1997).

Chronic effects are connected with high concentration of intracellular Ca+2 and inability of Ca+2-ATP-ase reorganize flux of calcium. This aspect leads to decrease of Ca+2 sensibility and diastolic dysfunction.

One of most toxic of Dox metabolites-doxorubicinol, can bound with intracellular Fe ions. In conditions of intracellular acidosis, doxorubicinol bounds to Fe+3, taking it from ferritin and activates Fe influx in the cell. Dox-Fe+3 complex metabolizes and form Dox-Fe+2 bound, what can oxidase lipids of organelles skeleton, proteins, DNA and form ROS. Due to significant lack of antioxidant system in cardiomyocytes, Dox-Fe+2 complexes can play key-role in Dox-induced apoptosis (Kwok and Richardson, 2003; 2004; Minotti, 1993; Minotti *et al*., 1999; 1995; Olson and Mushlin, 1990; Olson *et al*., 1988; Xu *et al*., 2005).

Dox interactions with mitochondria leads to cristae damage, membrane potential alteration, Ca ions storage in matrix, production of singlet oxygen. All these aspects are causing not only loss of mitochondria as functional unit, but activate them as apoptosis inductors (cytochrome C) (Minotti *et al*., 2004; Olson and Mushlin, 1990; Wang *et al*., 2001).

Dox metabolites alternates DNA and significantly damage tRNA and mRNA function. These changes cause depletion of synthetic activity of organelles and destabilization of energy producing (Minotti *et al*., 2004; Olson and Mushlin, 1990; Olson *et al*., 1988; Xu *et al*., 2005).

Retention of beta-oxidation intermediates inhibit normal flux of glycolysis. Glucose metabolites, stored in cytoplasm, can cause acidosis and pathological glycogen resynthesis (Minotti *et al*., 2004; Oleynikov *et al*., 2015).

Combination of all the components leads to local and general contractile dysfunction. Myocardial remodeling due to Dox-induced apoptosis can cause disturbances in electroactivity. Ion misbalance and developing remodeling characterize diastolic dysfunction.

In this study we are going to elucidate several differences in myocardial metabolism, histopathology in different cases of heart failure.

Animals, Materials, Methods

In this study, we studied 46 dogs of different sex, age and breed. All the dogs were patients of veterinary clinics “Pride” and “Champion”, Saint Petersburg, Russia. Groups were formed due to primary diagnosis: (1) Control (animals without cardiology problems, n = 14); (2) CDVD (animals with chronic degenerative valvulardisease, n = 16); (3) DCM (patients with idiopathic dilated cardiomyopathy and dilated cardiomyopathy–phenotype changes, n = 6); (4) DoxCM (dogs, treated with doxorubicin, n = 10).

The aim of this study is to elucidate some morphological and metabolic aspects of each nosologic units. Myocardium for research needs was obtained immediately after euthanasia on autopsy.

Studied animals were diagnosed by standard methods (clinical findings, blood analyzes, radiography, echocardiography, electrocardiography). Patients in control group, didn’t have diagnosed myocardial diseases and were euthanized due to low life quality. In other groups euthanasia were admitted in cases of end-stage and refractory heart failure and inability to sustain live functions or by owners will. Obtaining of samples were agreed with owners.

Materials were obtained right after death. Samples were taken from left ventricular apex. Material for histology fixed in neutral formalin and Bouin solution, for ultramicroscopic study-in glutaraldehyde, for biochemistry and ELISA-liquid nitrogen and stored in -25°C. All material were studied in two months, due to prevent significant loss in investigated substrates.

Histology samples were standardly prepared and stained with hematoxylin and eosin, metal hematoxylin, picrofuchsin and by Mallory. Ultramicroscopy studies were done in standard technic.

During biochemistry analyze we used standard kits for estimation of lactate concentration, activity of LDH and CK (Olvex Diagnosticum). In addition, we studied obtained samples to define concentrations of ATP and GLUT 4 protein by methods and reagents offered Cloud-Clone Corp. (USA).

Statistical analysis was made by using of Statistic 7.0, for non-parametric cases, we used Mann-Whitney criteria.

Results

During routine examination (echo-and electrocardiography) in control group there was no

evidences of myocardial dysfunction (data not presented). Data obtained from tissue biochemistry analyzes was taken as physiological. In addition, our results were compared with literature resources, what helped to define metabolism disturbances in different diseases.

Estimated concentrations of lactate and LDH in control group were taken as physiological level glycolytic myocardial tissue processes (Table 1).

LDH shows us activity and main path of carbohydrate metabolism. In control group, evaluated activity of this enzyme did not differ from commonly accepted data. We can assume that these measurements show transformation of unused pyruvate into lactate. Creatine kinase elucidate processes cooperation of creation, transport and concentration of macroergic phosphates and shows availability of energy supply. In this group, CK level characterizes unaltered energy storing. ATP concentrations are not very high in myocardium, but it compensates due to high activity of cytosolic enzymes. In the control group level of ATP was not different from observed data (Table 2).

Main glucose transporters in cardiomyocytes are GLUT 1 and GLUT4. Due to not very significant variability of GLUT 1 glucose transport in different circumstances and its role in basal support of glucose, in this study we investigated changes in GLUT4 proteins expression, in view of its flexibility regarding to tissue condition and insulin sensitivity. There is no conventional data of myocardium tissue concentration of GLUT4 protein, so we extrapolated it from skeletal muscle, where it expression was 142±39 ng\mg of wet tissue (Kim *et al*., 2005). In our study, we admitted GLUT4 protein concentration in alike diapason (Table 2).

Then we studied these parameters in animals with Chronic Degenerative Valvular Disease (CDVD). During statistical analyze we didn't define significant difference between control group and CDVD. This situation can be explained as CDVD even in terminal stages doesn't significant alter myocardial metabolism and it mostly compensated (Table 1 and 2). Therefore, we can define that main pathogenetic role in CDVD is played by volume overload. Decrease of systolic function is based on dysfunction of Frank-Starling mechanism, which plays significant role in organization of excessive amount of intravascular liquid.

It is necessary to admit that CDVD is usually associated with cardiomyocytes hypertrophy. This reaction is aimed on decreasing of left ventricular wall stress. While decompensation progress, adaptive gene expression is taking place in myocardial energy metabolism and protein synthesis, transforming it to fetal type. The most common changes include: Increasing of beta-MCH production, which contractility is not as flexible as it is in normal conditions; activation of CK BB-homoisomer synthesis, which has more affinity to ADP and is more effective in stabilizing of ATP concentration. One of the most significant parameter of fetal gene expression shifting is altering of GLUT1/GLUT4 ratio. This is based on 2 facts: (1) Altered insulin sensitivity leads to decrease of GLUT4 trafficking to membrane, but in this condition the whole amount of GLUT4 protein doesn't change (what is taking place in CDVD); (2) absolute decreasing of GLUT4 proteins and compensatory increase of insulin-insensitive GLUT1expression, what characterizes local insulin resistance on next stages.

In the end we have to admit that myocardial metabolism in CDVD stay mostly unchanged, but it should be keep in mind that it is based on several compensatory mechanisms and leads to decreased lability of energetic metabolism and lowering tolerance to exercises.

Then we should evaluate changes presented in myocardial tissue under anthracycline antibiotics treatment (Table 1 and 2).

Estimated lactate tissue concentration was significantly lower in compare with all other groups (control $p \leq 0.01$; CDVD $p \leq 0.01$; DCM $p \leq 0.05$). This situation is based on enzymatic disturbances under doxorubicin toxicity. Tissue lactate concentration characterize intensity of glycolysis substrate supplementation and wastes utilization. In this case, we find low activity of glucose incoming, due to decreased GLUT4 protein appearance (control $p \leq 0.01$; CDVD $p \leq 0.01$; DCM $p \geq 0.05$). Insufficiency of glucose supplementation leads to decreasing of intermedia metabolites production, mostly glucose-6-phosphate, which elucidate not only carbohydrate consuming, but also hexokinase activity, one of the weakest part of glycolysis chain.

In many available works were admitted that the main energy substrates during cardiomyopathy and heart failure progressing are shifting from free fatty acids to glucose. Hexokinase-is one of the threshold enzymes, taking part in carbohydrate metabolism regulation, during energy starvation hexokinase mRNA is significantly increase, bit its activity standstill, which is crucial limiting factor in glycolysis energy supply. Decreased glucose metabolism leads to lowering of pyruvate producing. This insufficiency has negative influence on TCA cycle, because even in normal conditions TCA intermedia metabolites (succinate, fumarate) leaks the cell, but in pathological situation and shortage of pyruvate makes this leakage irreversible and significant. Low concentrations of pyruvate leads to decreasing of lactate production. Therefore, we can say that low activity of LDH in case of DoxCM (control $p \leq 0.01$; CDVD $p \leq 0.01$; DCM $p \geq 0.05$) is based on 2 facts: (1) Shortage in substrate, due to glycolysis suppression; (2) altering effect of doxorubicin on NAD-depended dehydrogenase systems, this explanation also suitable for inability of pyruvate restoration by lactate and NAD metabolism.

Table 1. Myocardial biochemistry parameters

Group	Lact, µmol/g	LDH, U/g	CK, U\mg
Control (n = 14)	13,2±8,32	251,31±55,94	3,34±1,01
CDVD (n = 16)	13,44±4,22	264,58±47,42	3,93±0,55**
DCM (n = 6)	10,05±5,34++	94,87±42,74*+	1,36±0,24*+
DoxCM (n = 10)	8,84±4,7++	54,76±20,65*+	2,1±0,54*+""

* -p≤0,01 to control
** -p≤0,05 to control
+ -p≤0,01 to CDVD
++ -p≤0,05 to CDVD
" -p≤0,01 to DCM
"" -p≤0,05 to DCM

Table 2. ATP and GLUT4 tissue concentraions

Group	ATP, ng/g	GLUT 4, ng mg^{-1}
Control (n = 14)	3,37±0,67	138,68±88,81
CDVD (n = 16)	3,01±0,9	106,59±64,97
DCM (n = 6)	0,59±0,59*+	51,69±20,76*+
DoxCM (n = 10)	0,27±0,1*+	45,21±11,0*+

* -p≤0,01 to control
** -p≤0,05 to control
+ -p≤0,01 to CDVD
++ -p≤0,05 to CDVD
" -p≤0,01 to DCM
"" -p≤0,05 to DCM

It is known, there is an additive system, taking part in lactate supplementation for necessary pyruvate restoration-monocarboxylate transporters. However, in case of intracellular pH decreasing this structure is unable to transport available lactate, but let ketone bodies into cell.

It should be mentioned, there is one more imitating pyruvate utilization factor. Heart failure is usually associated with adrenergic stimulation for stroke volume compensation. Emission of catecholamine contribute to lipomobilization and increasing of serum free fatty acids concentration, which, due to their lipophilic nature, freely enter cardiomyocyte. Free fatty acid metabolites lead to PDH complex inhibition and abolish compensatory effects of glycolysis. In case of DoxCM it is complicated by the fact that Dox itself alters mitochondrial metabolism and leads to suppression of several transport systems, including carnitine palmitoiltransferase and NAD-depended enzymes. This blocks usage of absorbed free fatty acids in lipid beta-oxidation and ATP resynthesis. So, fatty acids storage in cytosol as triglycerides. Energy demand and blockage of fatty acids utilization leads to ATP-wasting cycles of mobilization-stabilization of cytosolic triglycerides, ceramides and products of lipid peroxidation storage.

Mitochondrial apparatus and energy metabolism alteration leads to CK system dysfunction. Though CK changed to BB-homoisomer Dox still inactivates it and, in parallel, leads to cell free creatine leakage due to decreased its phosphorylation. Doxorubicin coupled with mitochondrial membrane cardiolipines competitive blocks mitochondrial CK adhesion, alters connection with cytochrome apparatus. This leads to significant uncoupling of macroergic bounds form mitochondria to creatine transport. This reaction blocks creatine phosphorylation near mitochondria and then lowering of energy transport to contractile components due to suppressing cytosolic and mitochondrial CK.

Further, low GLUT4 protein concentration and CK alteration are leading to inhibition of PDH and its connection with hexokinase, triglyceride storage and local insulin resistance appearance.

Insufficiency of glycolysis substrates stimulates GLUT4 exocytosis and trafficking from intracellular storage places, but high concentrations of cytosolic triglycerides aborts GLUT 4 vesicles transport and membrane exocytosis. In addition, alternates signal pathways of insulin receptors, associated with straight PI-kinase inhibition, which controls GLUT4 mobilization.

Stress is associated with catecholamine emission, which stimulates hyperinsulinemia and hyperglycemia. Heart failure-is chronic stress, so it has own features-hyperglycemia is a rare fact, but hyperinsulinemia and tissue insulin resistance (in patients of 3 stage by NYHA classification) is mostly presented. This situation is accelerated by anthracycline. Doxorubicin stimulates corticosteroids secretion, decreases insulin receptors sensitivity, blocks AMPK phosphorylation and inhibit GLUT 4 trafficking to plasmolemma.

It is known, there are two forms of insulin receptors-A&B. A form-is characterized high affinity to insulin and in normal conditions represent 95% of all insulin receptors in brain and heart tissues. B form- is less affine to the hormone and often follow pathological situations, associated with long-term hyperinsulinemia.

The significant influence insulin resistance has on contractile components of myocardium, due to high amounts of GLUT4 in T-tubules. GLUT4 activity depression and decrease of their exposition in tubules leads to suppression of glucose supply and blocking of ATP synthesis for tubular ionic pumps.

Combination of presented alterations lead to decrease of ATP synthesis, which is markedly represented in group of DoxCM (control $p \leq 0.01$; CDVD $p \leq 0.01$; DCM $p \geq 0.05$).

Doxorubicin effects have influence on many vital functions of cardiomyocytes:

- Dox oxidizes components of mitochondrial DNA, alternates mitochondria and their enzymes resynthesis
- Affecting on cellular iron, creates ROS, hydroxide groups, which alone or in association with NO intercalate DNA, competitively couple with cofactors, necessary for electron transport chain
- Primarily stimulates release of Ca+2 ions from SR (acute faze, high risk of arrhythmias), then suppress activity of Ca+2-ATP-ase, which leads to storage of Ca+2 ions in cytosole and contracture of sarcomere
- Stimulates entrance and storage of Ca+2 in mitochondria, alternating its membrane potential and petrification
- In different ways alternates macroergic phosphates synthesis, what is highly notable in enzyme-ionic pump complex, which is strongly connected with glycolysis
- Leads to ionic pumps dysfunction, associated with myocardial edema, membrane potential alteration
- Stimulates insulin resistance appearance

Further we evaluated and estimated metabolic parameters in cases of DCM (Table 1 and 2). Received data shows significant changes in myocardial condition. We should note some aspects. Glycolysis in this situation is characterized with lower alternation, than in DoxCM group, this may be connected with less active damage of energy supplementation enzyme systems in absence of doxorubicin. GLUT4 protein concentration is much lower in compere with control and CDVD groups, but lactate, LDH, ATP measurements elucidate how cardiomyocytes trying to normalize their metabolism by glycolysis. It should be noted, DCM also characterized with insulin resistance, but it is a long-term process.

Discussion and Conclusion

Myocardium metabolism in control group characterized with normal glycolytic activity and more active oxidative phosphorylation, reflected in physiological ATP concentration. GLUT 4 protein concentration was alike to muscle concentration, so we extrapolated this data on myocardium and applied it as physiological state. We didn't define differences between membrane and cytosolic forms of GLUT 4 and their trafficking under insulin stimulation. However, due to normal ATP concentration and systolic function we admitted that GLUT 4 function was not altered.

In CDVD group, we defined statistically non-significant changes in studied parameters. This data characterized compensated state of myocardial energy metabolism. Nevertheless, referencing to available data, we can say that animals with decompensated state (retro- and anterograde heart failure), have 3 and higher stage in NYHA grades of failure, had myocardial IR (Friehs *et al*., 1990; Kolwitcz and Tian, 2011; Paternostro *et al*., 1999; Razeghi *et al*., 2001; Schlattner *et al*., 1998; Tokarska-Schlattner *et al*., 2005; Wallimann *et al*., 1999). Despite normal concentration of GLUT4 proteins, their activity and Ins response had been suppressed, what leaded to low flexibility of myocardial metabolism during exercises (exercise intolerance, dyspnea, muscle weakness).

Data obtained from DCM group characterized significant metabolic alterations. All investigated parameters were suppressed, except lactate concentration. This feature have to be connected with chronic development of this pathology and following energy substrate changing to glucose. Despite decreased GLUT 4 concentrations, level of produced lactate reflects ability to utilize carbohydrates for energy supplementation. Of course, it was not enough and energy producing is severely decreased, first of all due to mitochondria dysfunction. In comparison to our data there is an experimental work, what showed increasing in DCM myocardial GLUT 4 proteins concentration. We can't refute this possible compensatory reaction, but this findings could be connected with absence of significant decompensation due to little period of development. This means, in order to have stable genetic reaction and activation of fetal myocardium genetic pattern, activation of glycolytic path of energy supplementation and its exhaust myocardium needs some time.

In our study, DCM group didn't have marked compensatory AMPK-stimulated reactions, due to IR and cycling energy-wasting reactions of triglyceride mobilization-storage in cell (Minotti *et al*., 2004; Shibayama *et al*., 2015; van der Vusse *et al*., 2000). Low activity of CK, ATP concentration characterized myocardial energy starvation and following contractile dysfunction (Garcia *et al*., 1994; Hawkins, 1971; Johannsson *et al*., 1997; Osorio *et al*., 2002; Wilson *et al*., 1987). Combination of damaging factors leaded to massive cardiomyocytes apoptosis, adipose and connective tissue proliferation and fibrosis.

In DoxCM group, we observed significant decrease of all studied metabolic parameters. Obtained data characterized severe Dox-induced toxicity on cardiomyocytes in investigated cases.

In contrast to DCM, Dox toxicity develops in comparatively short period, so interpretation of received data is quite difficult. In case of DoxCM IR plays more important role on initial stages of heart failure, when utilization of FFA is increased, because of Dox-induced alteration of oxygen-depended components of myocardial energy metabolism. Increased consumption of FFA, mitochondrial dysfunction lead to cell triglyceride storage, suppression of PDH complex, disorders in GLUT 4 transport and membrane bound, Ins insensibility (de Lima Junior *et al*., 2016; Randle *et al*., 1963; Scwenk and Luiken, 2008). PDH block leads to inability to use carbohydrates as energy substrate and compensate

metabolic insufficiency (Tokarska-Schlattner *et al*., 2005; Wallimann *et al*., 1999). In addition, Dox toxicity causes impaired transport and enzyme proteins synthesis, negatively affect function of already presented proteins. This leads to degeneration of cell contractile components, vesicles formation and activation of phagolysosomes. In addition, Dox-induce: Retention of Na and stimulates cardiomyocyte edema: Change in cell-stored Fe activity and ROS generation; Ca+2 storage in sarcoplasm and following contracture, Ca+2 insensibility of myofilaments; increased retention of calcium ions in mitochondria leading to membrane potential disturbances and petrification.

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Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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Abbreviations

Acyl-coenzyme A-acyl-CoA
ADP-adenosine diphosphate
AMP-Adenosine monophosphate
AMPK-5' adenosine monophosphate-activated protein kinase
ATP-adenosine triphosphate
CDVD-chronic degenerative valvular disease
CK-creatine kinase
DCM-dilated cardiomyopathy
Dox-doxorubicin
DoxCM-doxorubicin cardiomyopathy
FFA-free fatty acids
GLUT-glucose transporters
Ins-insulin
IR-insulin resistance
LDH-lactate dehydrogenase
PCr-phosphocreatine
PDH-pyruvate dehydrogenase
ROS-reactive oxygen species
TCA-tricarboxylic acid cycle