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Metabolomics Of Metformin's Cardioprotective Effect In Acute Doxorubicin Induced- Cardiotoxicity In Rats

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ABSTRACT

Doxorubicin (DOX) is a powerful anticancer agent with sever cardiotoxic side effect which limits the clinical use. Metformin (MET) is antihyperglycemic drug with potential cardioprotective effect via AMP-activated protein kinase (AMPK) (increases fatty acid oxidation, decreases the production of ROS, maintaining energy homeostasis and apoptosis). Metabolomics technology deals with systematic study of chemical fingerprints of metabolite profiles. Different metabolic processes can be identified which will give information of any change in the metabolic profile of tissues as well as of biofluids after drug administration. This research designed to investigate MET cardioprotective effect against acute cardiotoxicity induced by DOX using metabolomics technology. Methods: Twenty four adult male wistar rats divided into four groups (6 animals each): control group (saline, i.p.); MET group (300mg/kg/day for 7dayes) by gavage; DOX group (20 mg/kg,i.p.) for acute induction of cardiotoxicity; Met + DOX group received DOX (20mg/kg i.p.) and Met (300 mg/kg/day, for 7 days, starting five days prior to DOX treatment) orally with gavage. Assessment of heart tissue metabolomics, serum MDA and GSH in addition to trichrome stain. Results: The results showed that pretreatment with MET (MET+DOX) significantly ($p<0.05$) reduced the level of acetic acid, cholesterol, palmitic acid, phosphoric acid, pyruvic acid, stearic acid, glucose, myo-inositol, alanine, lysine, and proline. Also, the level of arachidonic acid, hydroxybutyric acid, lactic acid, linoleic acid, oleic acid, oxalic acid, propionic acid, and galactose reduced after pretreatment with MET (MET+DOX). Additionally, (MET +DOX) resulted in significant decrease ($p<0.05$) in the level of serum MDA as well as significant ($p<0.05$) increase in serum GSH levels. In addition to significantly decreased collagen fiber production. Conclusion: It can be concluded that MET improved cardiac structure and function, as well as the metabolism of DOX-induced cardiotoxicity group, as it reduced the level of biomarkers associated with cardiotoxicity including (arachidonic, linoleic, oleic, acetic, stearic) acids, cholesterol ,leucine, glucose, mannose, myoinositol as well as hydroxybutyric acid. This indicates that MET induced a metabolic alterations, including the promotion of glycogenolysis, glycolysis, amino acid utilization and antioxidation. Additionally, MET improving energy metabolism and attenuating oxidative stress through suppression of serum MDA and increase the level of GSH as well as decrease fibrosis and structural changes.

Keywords: metabolomics, cardiotoxicity, doxorubicin, cardioprotection, metformin.

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INTRODUCTION

Doxorubicin (DOX), is potent anticancer drug from anthracycline family, have limited use because of its cardiotoxicity[1,2]. It derived from *Streptomyces* bacteria, a highly effective antitumor drug used for treatment different cancers like solid tumors, soft tissue sarcoma and hematological malignancies, but its organs toxicities (cardio-, nephro-, and hepatotoxicity) limits its use[3]. Cardiotoxicity induced by DOX is recognized by acute cardiac dysfunction, chronic cardiomyopathy and finally lead to congestive heart failure (HF). There is different kinds of molecular mechanisms like oxidative stress, inhibition of topoisomerase type II beta, alteration in energetics of mitochondria, the synthesis of protein and nucleic acid inhibited, apoptosis induction, interaction directly with the actin–myosin contractile proteins, hypothesis of anthracycline metabolite, platelet activating factor, intracellular calcium, prostaglandin alterations and etc., but the metabolic mechanism that occur and cause toxicity has not yet been fully identified. Cellular energetics have a critical role in cardiomyopathy induced by DOX[4–6].

Metformin (MET) is an oral antihyperglycemic agent, from biguanide family, widely used for diabetes mellitus(DM) type 2 patients. It decrease intestinal carbohydrates absorption, improving glucose peripheral uptake and utilization and enhanced insulin sensitivity [7,8]. It is approved to be effective in states associated with insulin resistance, like polycystic ovary syndrome[9], also it considered as an anticancer drug[10]. Furthermore, there are reports indicate that MET increases life span, also it improves gut flora [2][11]. MET produce its effects through AMPK dependent and independent pathways[12,13]. It is reported that this drug have cardioprotective effects in both hyperglycemic and normoglycemic subjects. Models of experimental animal of isolated heart failure and myocardial infarction have shown that MET increases myocardium tolerance to ischemic – reperfusion injury, decreases HF development after infraction by improving left ventricular ejection fraction, improves the outcomes of patients with advanced systolic HF and it may decrease DM complications[14,15].Through AMPK activation, MET able to reduce reactive oxygen specious (ROS)generation in animal models associated with HF, and protects myocardial cells from oxidative stress which is induced by TNFα and H2O2, accordingly, MET act as antioxidant agent and it have the ability to decrease lipid peroxidation. These cardioprotection and antioxidant

properties suggest that MET able to provide protection against DOX- cardiotoxicity[16,17].

Metabolomics is systematic study of chemical fingerprints of small-molecules complement (quantitatively and qualitatively) with in the biological system (culture supernatants, cells, tissues, and body fluids) which is also called metabolom, or metabolite profiles. These metaboloms are associated with a variety of metabolic processes present in a cell, organ, or organism[18,19]. Metabolomics is a developing method for a particular phenotyping of the endogenous and exogenous metabolite (usually, they are considered as cellular metabolic intermediates and products of less than 1 kDa in size) within a biological samples[20–22]. Metabolomics measures the response to perturbations like those related to the early diagnosis of disease and for the development and discovery of drugs[23–25]. It gives quantifiable information about body biochemical conditions in diseased and normal states[26]. Metabolomics aims to evaluate molecules having different physical properties such as, difference in polarity[24]. Metabolomics has been conducted to analyze DOX related toxicity biomarkers. Now, it is recognized as a technique widely used to advance the toxicology, also it become a significant method in study the mechanism of drugs that induce toxicity in metabolic level[27]. Metabolomics may identify cardiotoxicity early markers, and cardioprotective agents can be developed[2]. Serum biomarkers such as cardiac troponin T, do not reflect specifically the damage that occur in myocardium. Therefore, new biomarkers are needed for cardiac damage evaluation[28]. Though many urinary biomarkers have been identified from DOX systemic toxicity[6], myocardium tissue specific biomarkers need further study in order to provide a new understanding into the pathological processes of cardiomyopathy induced by DOX. Therefore, a gas chromatography–mass spectrometry(GC-MS) method depending on a metabolomics technique was developed. Additionally, the cardioprotective effects of MET and its mechanisms in reducing DOX-induced cardiotoxicity were also investigated by using a metabolomics approach.

METHODS

Animals: Twenty four adult male wistar rats (200-220gm), kept in cages with free access to food and water in the animal house/College of Pharmacy/Mustansiryiah University. The cages were placed in a quiet and temperature controlled room in which a 12:12-hour light-dark cycle was maintained. The rats were allowed a ten days acclimatization period before being used in experiments.

Study design: The rats divided randomly into four groups(6 rats/group). Control group (single dose of saline, 1 ml intraperitoneally (i.p.)). Met group; Met (300 mg/kg/day, every day for 7 days) with gavage. The acute DOX group received (20 mg/ kg single dose) i.p.. The Met + DOX group received DOX (20 mg/kg single dose) i.p. and Met (300 mg/kg/day, for 7 days, starting five days prior to DOX treatment) orally with gavage.

Induction of cardiotoxicity: Induction of cardiotoxicity carried out by the administration of DOX i.p. in a dose of 20 mg/kg as a single dose for acute cardiotoxicity induction[29].

Sample collection and preparation: At the end of the experiment, the rats were anesthetized by intraperitoneal administration of 50 mg/kg ketamine and 5 mg/kg xylazine[30]. Blood samples were collected directly from the Retro-orbital center by using heparinized capillary tubes[31], centrifuged at 2500x for 15 minutes for serum separation. The serum stored at -80°C for ELISA analysis. After getting the blood sample, the heart is immediately removed and washed with tap water then distilled water and rapidly stored in liquid nitrogen. Small portion of the heart tissue kept in 10% buffered neutral formalin to prepare paraffin embedded blocks for histopathological diagnosis.

Cardiac metabolomics

Extraction of metabolomics: Extraction of metabolites from left ventricle of the heart tissue were done by the method described by Gregor *et al.* (2012) using a chloroform-methanol procedure[32]. The stored heart tissues were thawed at room temperature for 5 minutes then ~100 mg of left ventricle heart tissue crashed and homogenized in 600 µL chloroform: methanol (1:2) previously cooled , using ceramic mortar and pestle then further pulverized in tissue homogenizer. Two hundred microliters of chloroform and then 200 µL deionized water (HPLC grade) was added to each sample. Samples were vortexed for 10 seconds, then centrifuged for 20 minutes at 4000 rpm in 4°C. The upper layer separated and dried in a fume hood under a stream of nitrogen gas. All extracted samples were stored at −20 ºC until required.

Derivatization of myocardium extracts: The stored dried extracted samples of the upper layer of the heart tissue warmed at room temperature 25°C for 10 minutes prior to derivatization. The samples were derivatized with 25 µl of methoxyamine hydrochloride in pyridine (Sigma Aldrich) (20 mg/ml)at 37°C for 90 min with agitation (40rpm) in water bath shaker. The second step of derivatization was performed by adding 40 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Sigma Aldrich) and incubation at 37°C for 30 min with agitation (40 rpm) in water bath shaker. Samples were subjected to GC/MS analysis directly after derivatization.

A quadruple GC-MS analysis condition[33]: 2 µL of each sample was Injected in split/splitless injector manually by a 10 µL syringe. The injection programs include, washing syringe before and after sample injection and removal of air bubbles by sample pumping and an air buffer for removal of sample from syringe after injection. The capillary column properties were phenyl- coated fused silica 35%, length of column 30 meter, film thickness were 0.32 mm I.D. and 0.25 µm.

The GC-MS operation procedure: The GC oven heated from 10°C/min for 60°C to 325°C, 1 min initial time and 10 min final time, running for 37.5 min and cooling down to 60°C. The ion source heat was adjusted to 220°C. Energy of electron was 70 eV. Splitless and split conditions were used for samples injection. Helium was used as carrier gas flushed out flow of 10.5 ml/min for 1 min, a saver run for 3 min at 20 ml/min rate. Mass Selective Detector (MSD) was put at 20 Hz signal data rate and set at 290°C for transfer line of the MSD. MS was operated on after 5.90 min of delay time of solvent.

Data analysis: Data analysis was carried out according to Fiehn and Kind, 2005[34,35]. Calculate the metabolomics in each sample represented by a GC-MS total ion chromatograms were identified (using the NIST mass spectral library) and the peak areas for each of the compound was determined by the relative level to control group according to the following equation:

Relative level of metabolite (fold change) = (sample areas-control area)/(control area)*100

Then convert the values to Log10 and the difference between metabolites determined by comparison the treated groups with the control groups.

Statistical analysis: Results were expressed as the mean ± the standard error of the mean (SEM). Statistical significance is indicated by (*) whenever the p value is < 0.05, and highly significant (**) whenever p < 0.01. One way ANOVA test, followed by a post hoc Tukey's multiple comparisons test was used to detect the significant difference between groups. GraphPad Prism 7 software was used in the statistical analysis.

RESULTS

Metabolomics detection of heart tissue:

The metabolomics detected from heart tissue in this test were 150 metabolites. Myocardium tissue metabolomics showed different metabolomics patterns (lipids, amino acids, carbohydrates, and metabolites) distributed between control and treated groups, elevated and reduced according to the activation and inhibition of certain metabolic pathway as shown in figure (1). The metabolomics identified in rat myocardium based on degree of metabolomics density in control, DOX, MET, and DOX+MET. The metabolomics data represented as the mean of percentage area is indicated as colored blocks in table (1).

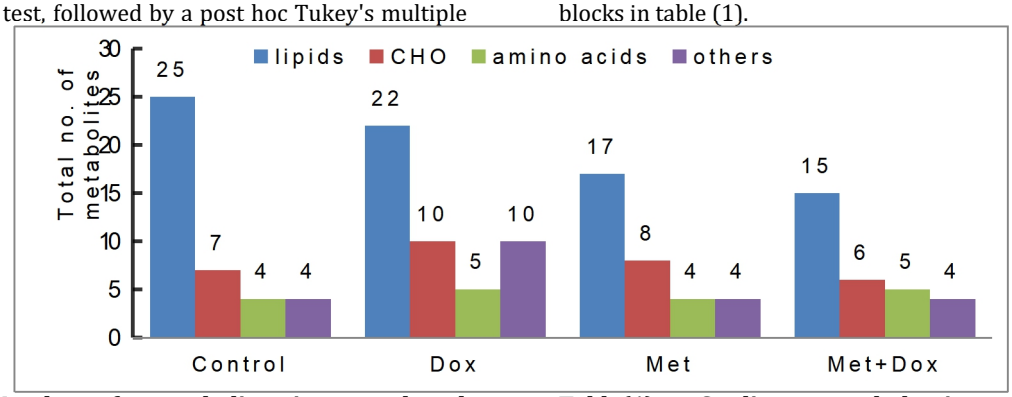

Figure 1: Number of metabolites in control and treated groups after treatment with Doxorubicin (DOX), Metformin(MET) and DOX+MET

Table(1): Cardiac metabolomics of control, Doxorubicin(DOX), Metformin(MET), MET+DOX (heart tissue derivatized by methoxyamine HCL in pyridine and MSTFA), data presented as the mean of percentage area ± slandered error

Metabolomes	Control	Doxorubicin	Metformin	Metformin+Doxorubicin
Acetic acid	0.1433±0.1	0.5767±0.11*	0.12±0.09#	0.1367±0.05#
Adipic acid	0.115	0.01	–	–
Arachidic acid	0.025	–	0.01	–
Arachidonic acid	0.2117±0.06	0.01±0.00*	0.205±0.06#	0.2033±0.01#
Hydroxybutyric acid	0.08833±0.01	0.01±0.00	0.235±0.07##	0.08667±0.01
Cholesterol	0.2033±0.04	0.4167±0.08*	0.008333±0.00*##	0.2±0.00#
Caprylic acid	0.01	–	–	–
Cyclohexanecarboxylic acid	–	0.28	0.19	–
2-Furoic acid	–	0.01	–	–
Gondoic acid	0.01	0.06	–	–
2-Hydroxyisocaproic acid	–	–	0.01	–
Hydroxypyruvic acid	–	0.02	–	–
Lactic acid	0.5217±0.22	0.01±0.00	1.658±0.7355	0.5±0.1
Lauric acid	–	0.01	–	–
Lignoceric acid	0.03	–	–	–
Linoleic acid	0.425±0.22	0.01167±0.00	0.3883±0.17	0.41±0.12
Malonic acid	–	0.71	–	–
Margaric acid	0.38	–	–	–
Methoxyacetic acid	–	–	–	0.17
Myristic acid	0.05	–	–	–

Oleic acid	1.805±0.80	0.09333±0.03*	1.742±0.90	1.68±0.63
Oxalic acid	0.26±0.06	0.095±0.01*	0.185±0.03	0.19±0.00
2-Oxovaleric acid	—	0.01	—	—
Palmitic acid	0.05333±0.03	0.22±0.07*	0.02±0.01##	0.03333±0.01#
Pentadecylic acid	0.01	—	—	—
Phenylacetic acid	—	—	—	0.12
Phosphoric acid	0.285±0.11	1.263±0.36*	0.01333±0.00##	0.3067±0.19#
Propanoic acid	1.127±0.39	0.3683±0.12	0.26±0.22	1.01±0.28
Pyruvic acid	0.06833±0.01	0.1817±0.01**	0.005±0.00**##	0.06667±0.01##
Ricinoleic acid	—	0.07	—	—
Succinic acid	—	—	0.12	—
Succioic acid	0.01	—	—	—
Stearic acid	0.09667±0.00	0.2883±0.03**	0.09±0.01##	0.1±0.00##
n-Tridecanoic acid	0.01	—	—	—
Terephthalic acid	0.26	—	—	—
Undecylenic acid	0.05	—	—	—
1-Deoxy-d-manitol	0.03667±0.01	0.06167±0.03	0.01±0.00	0.02±0.00
2-Deoxy-galactopyranose	—	0.03	—	—
d-Galactose	1.613±1.46	0.07±0.01	0.07667±0.06	0.1033±0.01
d-Glucose	0.1815±0.09	1.075±0.39*	0.01±0.00##	0.1867±0.02#
D-Glucosone	—	—	0.03	—
Erythrose	—	—	0.08	—
d-Mannose	0.27±0.05	0.2217±0.03	0.02333±0.01**#	0.1933±0.05
d-Mannitol	—	0.08	—	—
Glycerol	0.04±0.01	0.03333±0.02	0.7±0.27*#	0.07±0.00
Myo-Inositol	0.05±0.02	0.145±0.04*	0.015±0.00##	0.05333±0.01#
L-Rhamnose	0.01	0.03	—	—
2-Keto-d-gluconic acid	—	0.01	—	—
Alanine	19.02±0.15	59.5±2.06**	56.87±6.26**	25.67±1.02##
Glycine	—	—	—	0.01
L-Homoserine	—	9.165	—	—
l-Leucine	0.08667±0.01	0.01333±0.00**	0.01167±0.00**	0.01333±0.00**
L-Lysine	3.833±0.63	29.78±3.2**	25.82±3.35**	17.67±1.27**#
d-Proline	0.7333±0.20	4.067±0.13**	0.01±0.00##	0.78±0.48##

The data presented as percentage area mean ± SE of the mean, (*: p<0.05 significant, and **:p<0.01 highly significant) compare to control,
#: P<0.05, significant, and ##:p<0.01 highly significant, compared to DOX

Effect of metformin on serum malondialdehyde (MDA): Metformin showed a protective effect against MDA elevation in acute DOX toxicity. The descriptive statistics for MDA concentration which is represented as mean ± SE was significantly elevated in DOX group (0.2657±0.02 ng/ml) in comparison with the control and MET group (0.1058± 0.01, 0.071±0.00 ng/ml respectively; P < 0.001). The MDA concentration was significantly decreased in MET + DOX group in comparison with the DOX group (0.093±0.00 ng/ml; P < 0.001), figure (2).

Effect of metformin on serum glutathione (GSH): Metformin showed a protective effect against DOX induced cardiotoxicity by effecting serum level of GSH. The descriptive statistics for GSH concentration which is represented as mean ± SE was significantly reduced in DOX group (0.096±0.01ng/ml) in comparison with the control and MET group (0.12±0.01, 0.156±0.01ng/ml respectively; P < 0.001). The GSH concentration was significantly elevated in MET + DOX group in comparison with the DOX group (0.126±0.01ng/ml; P < 0.001), figure (3).

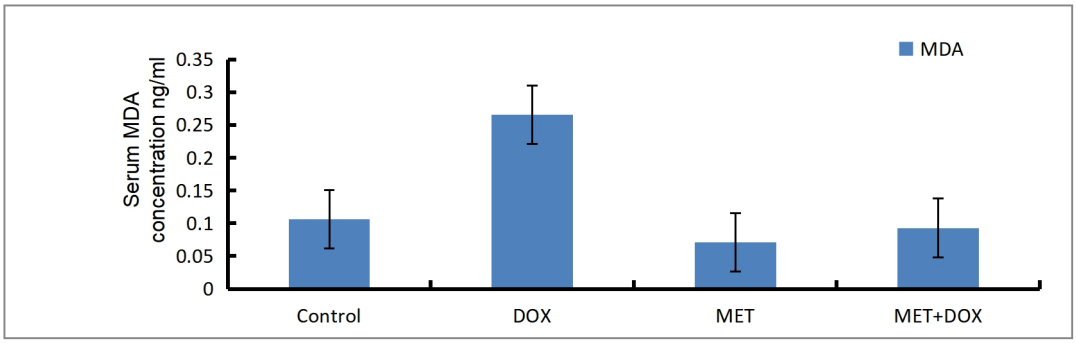

Figure 2: Effect of Metformin(MET) (300mg/kg) on serum malondialdehyde in acute Doxorubicin(DOX) cardiotoxicity (20mg/kg). Each value expressed as mean ± SE. The statistical analysis done by using one way ANOVA followed by Tukey test.

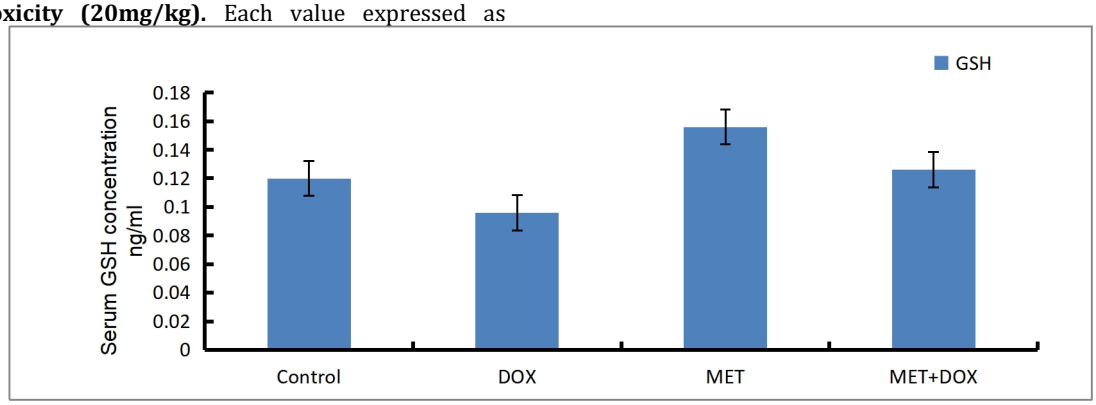

Figure 3: Effect of Metformin(MET) (300mg/kg) on serum glutathione (GSH) in acute Doxorubicin(DOX) cardiotoxicity (20mg/kg). Each value expressed as mean ± SE. The statistical analysis done by using one way ANOVA followed by Tukey test.

Effect of metformin on histological change by Trichrome stain:

MET decreased the collagenous fibers production in DOX cardiotoxicity. The cardiac tissue in DOX group showed mild increased in collagen fibers, while METC + DOX group was normal without any increase in collagen fibers as shown in figure (4).

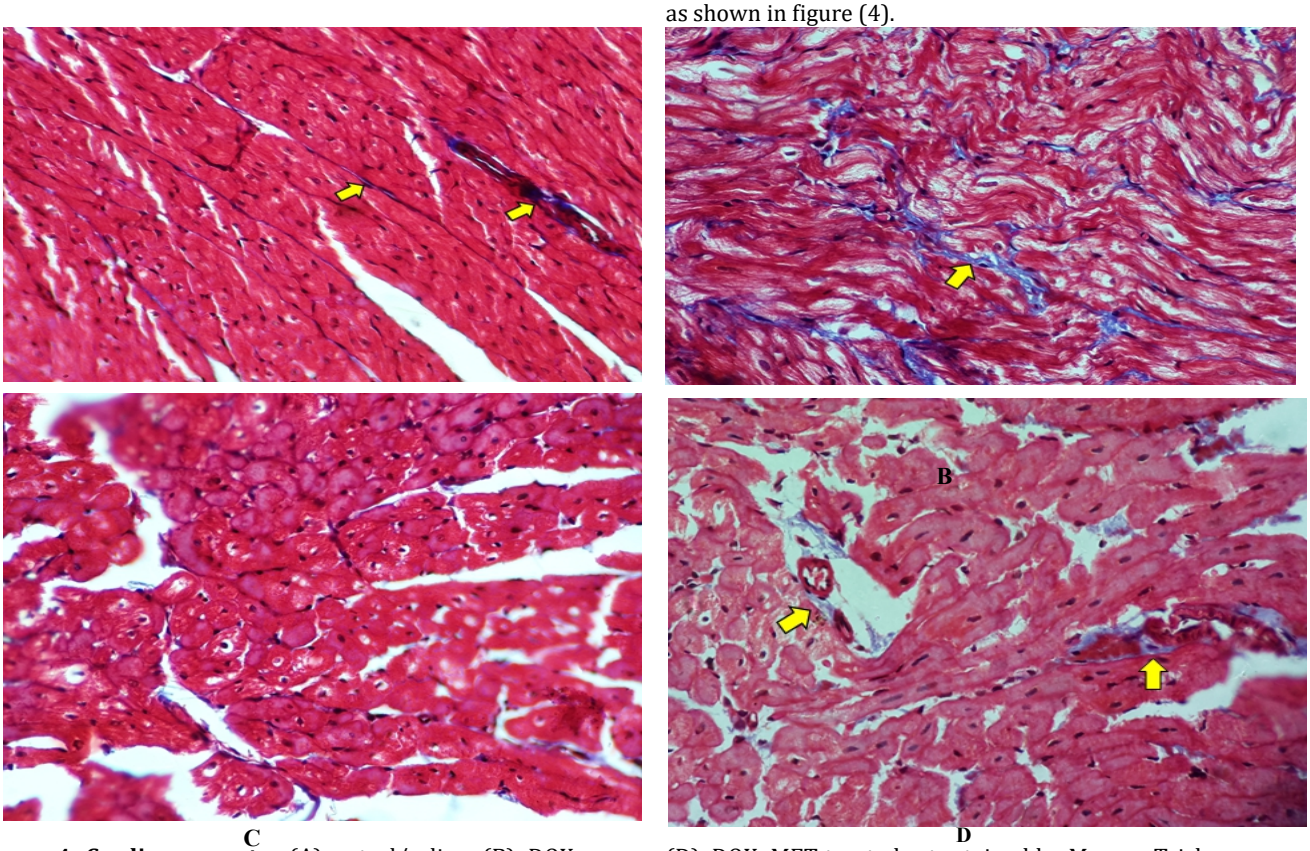

Figure 4: Cardiac myocytes (A)control/saline; (B), DOX (20mg/kg) treated; (C), MET (300mg//kg) treated and (D), DOX+MET treated rats stained by Masson Trichrome stain, X400.

DISCUSSION

Metabolomics is now consider as an effective research field in studying cardiac metabolism which have been correlated to many diseases of the myocardium. Additionally much interest appeared in the use of metabolomics to identify biomarkers in CVDs. Meanwhile, the development in technology of NMR and GC-MS helped remarkably in displaying the pattern of pathophysiology of many CVDs[36]. In this study, metabolomics analysis based on GC-MS was used to display the metabolomics profile of myocardium with the acute toxicity induced by DOX, and in the presence of MET as a cardioprotective agent. The present study identified potential biomarkers related to the myocardial energy metabolism disturbance mainly involving glycolysis, metabolism of lipids, and metabolism of some amino acids. Also, it was confirmed that MET could produce cardioprotection intervening in some metabolic pathways, such as glycolysis, metabolism of lipids, and metabolism of some amino acids, which might be plotted by restoring some biomarkers alterations found in this research. Metabolic profiling based on GC-MS considered as flexible approach to recognize pharmaceutical compounds with possible physiological toxicity. Moreover, metabolomics may identify metabolites as biomarkers of toxicity in predictive preclinical toxicity screening. From these metabolites, lactate and glucose are associated with glycolysis, give indication about modulation of the glycolytic pathway referring to a defect in TCA cycle. In this study lactate level reduced in DOX treated group(table (1)). It is possible that this reduction accompanied with the enzymes regulation in the heart. Lactate dehydrogenase B (LDHB), (subtype of lactate dehydrogenase) mainly present in myocardium, was found recently to be raised in the cardiomyocytes treated with DOX, which could result in reduction of lactate by the conversion of lactate to pyruvate [37]. The level of both lactic acid and pyruvic acid returned to normal when MET co-administered with DOX (table 1), this could illustrate that MET co-administration might made the heart took enough energy during stressed conditions by balancing the level of TCA cycle intermediates and increased the ability of the mitochondria to produce energy. Glucose shown to be increased significantly ($p<0.05$) (table 1) in DOX group, this elevation might be result from the decreased glucose and other sugars utilization (ex. galactose) in conditions associated with myocardial stress [37,38]. The level of glucose improved after the use of MET with DOX (table 1), which mean that the glycolysis process might be balance with the fatty acids oxidation in order to produce enough energy to meet the requirement of the heart to work normally during stress conditions. Mannose is sugar considered as a marker of oxidative stress [39,40], after MET treatment the level of mannose reduced since MET activates mannose-selective transport system, this suggests that AMP-activated protein kinase may be a regulator of mannose metabolism which made MET decrease the oxidative stress induced in myocardium after DOX treatment[41].

Lipid metabolism has an important role in cardiomyopathy induced by DOX[42], due to the importance of lipids in the metabolic activity of the heart which act as a source of energy. The level of saturated fatty acid (stearic acid) found to be increased significantly ($p<0.05$) in DOX treated rats(table1), this increase may indicate that there was inhibition in the ß-oxidation of the saturated fatty acids. Mainly because of inhibition of TAC cycle (citrate cycle) and oxidative phosphorylation due to DOX accumulation in the heart, as a result the acyl-CoA and NADH also accumulated[37]. However, the level of linoleic acid, arachidonic acid, and oleic acid were found to be decreased significantly ($p<0.05$) (table 1) in DOX group, possibly because of the peroxidation of the unsaturated fatty acids in the presence of DOX, leading to excessive oxidation damage on the mitochondria of the myocardium[43,44]. As noted in table (1) the co-administration of MET with DOX largely improve the metabolism of fatty acids, which might be due to improvement in lipid metabolism and there was reduction in lipid peroxidation leading to improvement in energy metabolism. The normally beating heart required high energy supply which is largely depend on the supply of pyruvate and fatty-acyl coenzyme A[45]. In the present study the level of pyruvate and acetate increased significantly ($p<0.05$) in DOX group. Acetate is the main source of acetyl-CoA and it regulate energy in the mammalian cells[46]. In the mitochondria, acetyl-CoA derived from pyruvate and acetate, enters into the TCA cycle in order to meet energy requirements[47]. DOX reduce the uptake of pyruvate and acetate by the cells, which reduces pyruvate and acetate metabolism by the mitochondria to produce energy. In this context, Andreadou et al. (2009) approved that DOX significantly increased acetate levels in myocardium tissue, the authors suggested that these metabolites considered as biomarkers for DOX cardiotoxicity[5]. The level of cholesterol was shown to be elevated significantly ($p<0.05$) (table1) in DOX group. Lipolysis rate could be reduced or blocked by DOX as approved by the previous studies, suggesting that cholesterol accumulation was a characteristic of cardiotoxicity induced by DOX[37,48]. The level of cholesterol decreased significantly ($p<0.05$) (table1) MET treated group, because MET could reduce accumulation of cholesterol in myocardial tissue which improve energy metabolism, as a result it can be safely used for treatment of cardiac dysfunction induced by DOX[49,50].

In present study leucine level found to be reduced significantly ($p<0.05$) (table 1) in DOX group. It is one of branched-chain amino acids (BCAAs), may be considered as an important alternative substrate of energy for the heart in myocardial ischemia. It appears that the reduction in the production of ATP, due to the ß-oxidation inhibition of fatty acids and inhibition of citrate cycle induced by DOX treatment, could lead to the use of BCAAs as energy compensation[27,28,51]. BCAAs proposed to be cardioprotective; they are considered as a source of acetyl-CoA, attributed to the production of NADH and FADH, and are involved in glutamine and glutamate conversion to free radical scavenger (GSH)[52,53], and this might be a reason why BCAA decreased after DOX administration. GC-MS spectra also exhibited the changes in the metabolism of other amino acids, where the level of L-Lysine, L-alanine, and L-proline were observed to be increased significantly ($p<0.05$) (table 1) in DOX treated group, this could be due to the oxidative stress induced by DOX lead to the metabolic alteration of α-amino acids in order to meet myocardial energy requirement[28,37]. In MET+DOX treated group, amino acids levels located between normal (proline) to high level (lysine, alanine) as showed in table(1), this could occur to regulate energy requirement of the heart under stressed conditions, which might make the heart work somewhat normally

through increased gluconeogenesis by MET in myocardium and reverse the metabolic alteration that occur in heart during DOX treatment. In addition, the level of phosphate found to be elevated significantly ($p<0.05$) (table 1) in DOX treated group, since DOX were usually associated with the depletion of ATP and phosphate accumulation[3,54]. In general, the mitochondrial dysfunction and ATP depletion might be responsible for the DOX cardiotoxicity [55]. In the present study the level of hydroxybutyric acid was decreased table(1) in DOX group. Hydroxybutyric acid is a ketone body that have the ability to produce protection to the heart in situations associated with oxidative stress like in case of heart failure and cardiotoxicity. ketone body oxidation increased in cases associated with myocardial stress, and increased ketone body utilization decreased oxidative stress and protected against cardiotoxicity induced by DOX[56], this indicate that 3-hydroxybutyrate protect the myocardium from DOX-induced oxidative stress[28]. Hydroxybutyric acid level was increased (table 1) with the use of MET treatment and return to normal in MET+DOX treated group. MET increase the production of hydroxybutyric acid mainly in tissues and might made its level return to normal during detoxification or oxidative stress as well as provide energy homeostasis [40,57]. The level of myoinositol found to be increased after DOX treatment whereas reduced +(table 1) in MET treated group and return to normal in MET+DOX group. This could occur since myoinositol metabolism were closely related to the citric acid cycle, and it is involved in the activation of protein kinase C (PKC), which plays an important role in glucose metabolism by which the myocardial dysfunction after DOX cardiotoxicity improved[40,58].

In present study the level of MDA and GSH were found to be significantly changed ($p<0.05$) in DOX treated group when compared with the control and MET treated group and then the level of these biomarkers return to control level in MET+DOX group. This could occur as a result of the oxidative stress induced by DOX which may lead to the production of free radicals which result in overwhelming the limited capacity of heart to detoxify free radicals like $O_2^{\cdot-}$, $NO^{\cdot}$, resulting in extensive oxidative damage to myocardial cells and reducing the level of cardioprotective detoxifying agent GSH, followed by enhancement of lipid peroxidation resulting in increased MDA level[59,60]. MET treatment not only improved DOX-induced cardiac dysfunction, but also decreased oxidative stress by preventing the protein oxidation and lipid peroxidation, as well as rising antioxidant restoration by increasing serum GSH. Collectively these remarks powerfully advocate MET as a potent antioxidant agent and could exert cardioprotective effects by alleviating DOX-induced oxidative stress[61,62].

In this study, the DOX+MET treated group showed mild edema, and cytoplasmic vacuoles, and the myofibrillar structure with striation well preserved in compare with DOX group. These finding agreed with Ashour *et.al. (2012*), that reported oral therapy of MET (50 mg/kg and 500 mg/kg) removed histopathological changes produced by DOX (18 mg/kg) cumulative dose, so exerting a cardioprotective effects[7]. Janeesh *et.al (2014),* showed that DOX have changes on normal morphology of cardiomyocyte including necrosis, myofibrillar loss, vacuolization, and mononuclear cells infiltration due to the action of oxidative stress that considered as an indication for cardiac injury and dysfunction[63]. Bahadir *et. al. (2014),* showed that DOX 15mg/kg (cumulative dose) causing myocyte edema, myocyte vacuolization and loss of myofibrils[64]. Sheta *et.al.2016,* showed that MET treatment reduce DOX induced alterations in myocardium, reported by normal appearance of muscle fibers, less widening of the interstitial space, no myocyte degeneration, moderate interstitial cellular infiltration, and normal location of nuclei[65].

CONCLUSION

From this study, it could be concluded that DOX administration caused cardiotoxicity that it produce significant cardiac metabolic perturbations observed in rats myocardium, which involved the unsaturated fatty acids that undergo peroxidation particularly arachidonic acid, linoleic acid, and oleic acid as a result of increased oxidative stress produced by DOX. Also, DOX reduce the uptake of acetic acid (saturated fatty acid) by the cells, as a result, the ability of the mitochondria reduced to metabolize acetate to produce energy. DOX cardiotoxicity characterized by accumulation of cholesterol in myocardium. Glucose consumption reduced in heart tissue due to DOX oxidative stress, whereas BCAAs (leucine) consumption increased in this situation as the heart depended on these amino acids as a source of energy. So these metabolites collectively expected to be biomarkers of cardiotoxicity induced by DOX. MET co-administration markedly reduce cardiotoxicity induced by DOX and it is conceder as a useful agent in cardiac damage induced by DOX, as it improve metabolic perturbations and cardiac functions of rat cardiomyopathy induced by DOX. The possible mechanism by which MET produces these effects may be attributed to energy metabolism improvement by increase entry of glucose into the cell, improve fatty acid oxidation (acetic acid, stearic acid), increase protein utilization (like leucine) and oxidative stress attenuation (increased hydroxybutyric acid level). So these metabolites collectively expected to be new cardioprotective biomarkers induced by MET against cardiotoxicity induced by DOX. MET co-administration largely reduce myocardial damage produced as a result of DOX-induced oxidative stress by reducing the level of MDA (common marker of oxidative stress) and increase the level of GSH (endogenous antioxidant) since MET have the ability to reduce ROS by attenuation of lipid peroxidation and protein oxidation. MET co-administration markedly reduced the collagenous fibers and other histopathological changes in myocardial tissue. So, the current study showed that MET have a good cardioprotective effect against DOX cardiotoxicity through various mechanisms, that discovered new cardioprotective biomarkers produced by MET in the presence of a drug that causes acute myocardial toxicity like DOX by metabolomics technology.

REFERENCES

1. Li Y, Ju L, Hou Z, Deng H, Zhang Z, Wang L, et al. Screening, verification, and optimization of biomarkers for early prediction of cardiotoxicity based on metabolomics. J Proteome Res. 2015 Jun 5;14(6):2437–45.
2. Madonna R. Early Diagnosis and Prediction of Anticancer Drug-induced Cardiotoxicity: From Cardiac Imaging to 'Omics' Technologies. Rev Espanola Cardiol Engl Ed. 2017 Jul;70(7):576–82.

3. Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL. Doxorubicin-induced cardiomyopathy: From molecular mechanisms to therapeutic strategies. J Mol Cell Cardiol. 2012 Jun 1;52(6):1213–25.
4. Liu X, Romero IL, Litchfield LM, Lengyel E, Locasale JW. Metformin Targets Central Carbon Metabolism and Reveals Mitochondrial Requirements in Human Cancers. Cell Metab. 2016 08;24(5):728–39.
5. Chaudhari U, Ellis JK, Wagh V, Nemade H, Hescheler J, Keun HC, et al. Metabolite signatures of doxorubicin induced toxicity in human induced pluripotent stem cell-derived cardiomyocytes. Amino Acids. 2017 Dec;49(12):1955–63.
6. Wang J, Reijmers T, Chen L, Van Der Heijden R, Wang M, Peng S, et al. Systems toxicology study of doxorubicin on rats using ultra performance liquid chromatography coupled with mass spectrometry based metabolomics. Metabolomics. 2009 Dec;5(4):407–18.
7. Ashour AE, Sayed-Ahmed MM, Abd-Allah AR, Korashy HM, Maayah ZH, Alkhalidi H, et al. Metformin Rescues the Myocardium from Doxorubicin-Induced Energy Starvation and Mitochondrial Damage in Rats [Internet]. Oxidative Medicine and Cellular Longevity. 2012 [cited 2019 Sep 8]. Available from: https://www.hindawi.com/journals/omcl/2012/434195/
8. Batchuluun B, Sonoda N, Takayanagi R, Inoguchi T. The Cardiovascular Effects of Metformin: Conventional and New Insights. J Endocrinol Diabetes Obes. 2014 May 28;2:1035.
9. Janić M, Volčanšek Š, Lunder M, Janež A. Metformin: from mechanisms of action to advanced clinical use. In 2017.
10. Barzilai N, Crandall JP, Kritchevsky SB, Espeland MA. Metformin as a Tool to Target Aging. Cell Metab. 2016 14;23(6):1060–5.
11. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Mannerås-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017 Jul;23(7):850–8.
12. Griss T, Vincent EE, Egnatchik R, Chen J, Ma EH, Faubert B, et al. Metformin Antagonizes Cancer Cell Proliferation by Suppressing Mitochondrial-Dependent Biosynthesis. PLoS Biol. 2015 Dec;13(12):e1002309.
13. Yan M, Qi H, Xia T, Zhao X, Wang W, Wang Z, et al. Metabolomics profiling of metformin-mediated metabolic reprogramming bypassing AMPKα. Metabolism. 2019;91:18–29.
14. Argun M, Üzüm K, Sönmez MF, Özyurt A, Derya K, Çilenk KT, et al. Cardioprotective effect of metformin against doxorubicin cardiotoxicity in rats. Anatol J Cardiol. 2016;16(4):234–41.
15. Kamel M, Omar El Farouk L, Sayed Osman A, Khorshid O, EL Shabrawy Abdo M. Comparative Study of the Protective Effect of Metformin and Sitagliptin against Doxorubicin-Induced Cardiotoxicity in Rats. Clin Pharmacol Biopharm. 2017 Jan 1;06.
16. Kobashigawa LC, Xu YC, Padbury JF, Tseng Y-T, Yano N. Metformin protects cardiomyocyte from doxorubicin induced cytotoxicity through an AMP-activated protein kinase dependent signaling pathway: an in vitro study. PloS One. 2014;9(8):e104888.
17. Bhave KA, Pandit PR, Ved JK, Amonkar G. Evaluation of Cardioprotective Effect of Metformin in Isoproterenol Induced Myocardial Injury in Rats. In 2016.
18. Senn T, Hazen SL, Tang WHW. Translating metabolomics to cardiovascular biomarkers. Prog Cardiovasc Dis. 2012 Aug;55(1):70–6.
19. Wang J, Wang C, Liu H, Qi H, Chen H, Wen J. Metabolomics assisted metabolic network modeling and network wide analysis of metabolites in microbiology. Crit Rev Biotechnol. 2018 Oct 3;38(7):1106–20.
20. Rankin NJ, Preiss D, Welsh P, Sattar N. Applying metabolomics to cardiometabolic intervention studies and trials: past experiences and a roadmap for the future. Int J Epidemiol. 2016;45(5):1351–71.
21. Beger RD, Dunn W, Schmidt MA, Gross SS, Kirwan JA, Cascante M, et al. Metabolomics enables precision medicine: 'A White Paper, Community Perspective'. Metabolomics Off J Metabolomic Soc. 2016;12(10):149.
22. Dunn WB, Goodacre R, Neyses L, Mamas M. Integration of metabolomics in heart disease and diabetes research: current achievements and future outlook. Bioanalysis. 2011 Oct;3(19):2205–22.
23. Kasture VS, Musmade DS, Vakte MB, Sonawane SB, Patil PP. METABOLOMICS: CURRENT TECHNOLOGIES AND FUTURE TRENDS. 2012;12.
24. Clish CB. Metabolomics: an emerging but powerful tool for precision medicine. Cold Spring Harb Mol Case Stud [Internet]. 2015 Oct [cited 2019 Sep 7];1(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4850886/
25. Trivedi DK, Hollywood KA, Goodacre R. Metabolomics for the masses: The future of metabolomics in a personalized world. New Horiz Transl Med. 2017 Mar;3(6):294–305.
26. Suhre K, Raffler J, Kastenmüller G. Biochemical insights from population studies with genetics and metabolomics. Arch Biochem Biophys. 2016 Jan 1;589:168–76.
27. Li Y, Zhang X, Zhou H, Fan S, Wang Y, Wang L, et al. Toxicity analysis of doxorubicin using plasma metabolomics technology based on rapid resolution liquid chromatography coupled with quadruple-time-of-flight mass spectrometry. Anal Methods. 2014 Jul 10;6(15):5909–17.
28. QuanJun Y, GenJin Y, LiLi W, YongLong H, Yan H, Jie L, et al. Protective Effects of Dexrazoxane against Doxorubicin-Induced Cardiotoxicity: A Metabolomic Study. PLoS ONE [Internet]. 2017 Jan 10 [cited 2019 Sep 7];12(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5224977/
29. Polegato BF, Minicucci MF, Azevedo PS, Carvalho RF, Chiuso-Minicucci F, Pereira EJ, et al. Acute Doxorubicin-Induced Cardiotoxicity is Associated with Matrix Metalloproteinase-2 Alterations in Rats. Cell Physiol Biochem. 2015;35(5):1924–33.
30. Struck MB, Andrutis KA, Ramirez HE, Battles AH. Effect of a short-term fast on ketamine-xylazine

anesthesia in rats. J Am Assoc Lab Anim Sci JAALAS. 2011 May;50(3):344–8.
31. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother. 2010;1(2):87–93.
32. McCombie G, Medina-Gomez G, Lelliott CJ, Vidal-Puig A, Griffin JL. Metabolomic and Lipidomic Analysis of the Heart of Peroxisome Proliferator-Activated Receptor-γ Coactivator 1-β Knock Out Mice on a High Fat Diet. Metabolites. 2012 Jun 18;2(2):366–81.
33. Kind T, Wohlgemuth G, Lee DY, Lu Y, Palazoglu M, Shahbaz S, et al. FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. Anal Chem. 2009 Dec 15;81(24):10038–48.
34. Fiehn O, Kind T. Metabolite profiling in blood plasma. Methods Mol Biol Clifton NJ. 2007;358:3–17.
35. Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, et al. Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol. 2005 May;138(1):304–18.
36. Deidda M, Mercurio V, Cuomo A, Noto A, Mercuro G, Cadeddu Dessalvi C. Metabolomic Perspectives in Antiblastic Cardiotoxicity and Cardioprotection. Int J Mol Sci [Internet]. 2019 Oct 4 [cited 2021 Jan 3];20(19). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6801977/
37. Tan G, Lou Z, Liao W, Zhu Z, Dong X, Zhang W, et al. Potential biomarkers in mouse myocardium of doxorubicin-induced cardiomyopathy: a metabonomic method and its application. PloS One. 2011;6(11):e27683.
38. Geng C, Cui C, Wang C, Lu S, Zhang M, Chen D, et al. Systematic Evaluations of Doxorubicin-Induced Toxicity in Rats Based on Metabolomics. ACS Omega [Internet]. 2020 Dec 28 [cited 2021 Jan 3]; Available from: https://doi.org/10.1021/acsomega.0c04677
39. Hameed A, Iqbal N, Malik S. Effect of D-mannose on antioxidant defense and oxidative processes in etiolated wheat coleoptiles. Acta Physiol Plant. 2014 Jan 1;36.
40. den Ouden H, Pellis L, Rutten GEHM, Geerars-van Vonderen IK, Rubingh CM, van Ommen B, et al. Metabolomic biomarkers for personalised glucose lowering drugs treatment in type 2 diabetes. Metabolomics [Internet]. 2016 [cited 2019 Sep 7];12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4703625/
41. Shang J, Lehrman MA. Metformin-stimulated mannose transport in dermal fibroblasts. J Biol Chem. 2004 Mar 12;279(11):9703–12.
42. Hong YM, Kim HS, Yoon H-R. Serum lipid and fatty acid profiles in adriamycin-treated rats after administration of L-carnitine. Pediatr Res. 2002 Feb;51(2):249–55.
43. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.
44. Wallace Kendall B., Sardão Vilma A., Oliveira Paulo J. Mitochondrial Determinants of Doxorubicin-Induced Cardiomyopathy. Circ Res. 2020 Mar 27;126(7):926–41.
45. Kolwicz SC, Purohit S, Tian R. Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. Circ Res. 2013 Aug 16;113(5):603–16.
46. Shimazu T, Hirschey MD, Huang J-Y, Ho LTY, Verdin E. Acetate metabolism and aging: An emerging connection. Mech Ageing Dev. 2010 Aug;131(7–8):511–6.
47. Gaspar JA, Doss MX, Hengstler JG, Cadenas C, Hescheler J, Sachinidis A. Unique metabolic features of stem cells, cardiomyocytes, and their progenitors. Circ Res. 2014 Apr 11;114(8):1346–60.
48. Chen Y, Tang Y, Zhang Y-C, Huang X-H, Xie Y-Q, Xiang Y. A Metabolomic Study of Rats with Doxorubicin-Induced Cardiomyopathy and Shengmai Injection Treatment. PLOS ONE. 2015 May 4;10(5):e0125209.
49. Riera-Borrull M, García-Heredia A, Fernández-Arroyo S, Hernández-Aguilera A, Cabré N, Cuyàs E, et al. Metformin Potentiates the Benefits of Dietary Restraint: A Metabolomic Study. Int J Mol Sci. 2017 Oct 28;18(11).
50. Wilmanns JC, Pandey R, Hon O, Chandran A, Schilling JM, Forte E, et al. Metformin intervention prevents cardiac dysfunction in a murine model of adult congenital heart disease. Mol Metab. 2019;20:102–14.
51. Andreadou I, Mikros E, Ioannidis K, Sigala F, Naka K, Kostidis S, et al. Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. J Mol Cell Cardiol. 2014 Apr;69:4–16.
52. Drake KJ, Sidorov VY, McGuinness OP, Wasserman DH, Wikswo JP. Amino acids as metabolic substrates during cardiac ischemia. Exp Biol Med Maywood NJ. 2012 Dec;237(12):1369–78.
53. Nejdl L, Sochor J, Zitka O, Cernei N, Ruttkay-Nedecky B, Kopel P, et al. Spectrometric and Chromatographic Study of Reactive Oxidants Hypochlorous and Hypobromous Acids and Their Interactions with Taurine. Chromatographia. 2013 Apr 1;76(7):363–73.
54. Pointon AV, Walker TM, Phillips KM, Luo J, Riley J, Zhang S-D, et al. Doxorubicin in vivo rapidly alters expression and translation of myocardial electron transport chain genes, leads to ATP loss and caspase 3 activation. PloS One. 2010 Sep 15;5(9):e12733.
55. Rosca MG, Tandler B, Hoppel CL. Mitochondria in cardiac hypertrophy and heart failure. J Mol Cell Cardiol. 2013 Feb;55:31–41.
56. Uchihashi M, Hoshino A, Okawa Y, Ariyoshi M, Kaimoto S, Tateishi S, et al. Cardiac-Specific Bdh1 Overexpression Ameliorates Oxidative Stress and Cardiac Remodeling in Pressure Overload-Induced Heart Failure. Circ Heart Fail. 2017 Dec;10(12).
57. Cadeddu C, Deidda M, Nocco S, Locci E, Cossu E, Baroni MG, et al. Effects of Metformin Treatment on Myocardial and Endothelial Function in Insulin Resistance Patients: A Metabolomic Study. In 2013.
58. Park J-E, Jeong G-H, Lee I-K, Yoon Y-R, Liu K-H, Gu N, et al. A Pharmacometabolomic Approach to

Predict Response to Metformin in Early-Phase Type 2 Diabetes Mellitus Patients. Mol Basel Switz. 2018 Jun 29;23(7).
59. Hyun Kim S, Kim K-J, Kim J-H, Kwak J-H, Song H, Young Cho J, et al. Comparision of doxorubicin-induced cardiotoxicity in the ICR mice of different sources. Lab Anim Res. 2017 Jun 1;33:165.
60. Wu Z, Zhao X, Miyamoto A, Zhao S, Liu C, Zheng W, et al. Effects of steroidal saponins extract from Ophiopogon japonicus root ameliorates doxorubicin-induced chronic heart failure by inhibiting oxidative stress and inflammatory response. Pharm Biol. 2019 Dec;57(1):176–83.
61. Sadeghi H, Jahanbazi F, Sadeghi H, Omidifar N, Alipoor B, Kokhdan EP, et al. Metformin attenuates oxidative stress and liver damage after bile duct ligation in rats. Res Pharm Sci. 2019 Mar 6;14(2):122–9.
62. Paseban M, Mohebbati R, Niazmand S, Sathyapalan T, Sahebkar A. Comparison of the Neuroprotective Effects of Aspirin, Atorvastatin, Captopril and Metformin in Diabetes Mellitus. Biomolecules. 2019 27;9(4).
63. Janeesh PA, Abraham A. Robinin modulates doxorubicin-induced cardiac apoptosis by TGF-β1 signaling pathway in Sprague Dawley rats. Biomed Pharmacother Biomedecine Pharmacother. 2014 Oct;68(8):989–98.
64. Bahadır A, Kurucu N, Kadıoğlu M, Yenilme E. The Role of Nitric Oxide in Doxorubicin-Induced Cardiotoxicity: Experimental Study. Turk J Hematol. 2014 Mar;31(1):68–74.
65. Sheta A, Elsakkar M, Hamza M, Solaiman A. Effect of metformin and sitagliptin on doxorubicin-induced cardiotoxicity in adult male albino rats. Hum Exp Toxicol. 2016 Jan 28;35.