Lipidomics Application to Explain Acute Cardiotoxicity Induced by Doxorubicin

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Abstract:

Doxorubicin (DOX) induced cardiotoxicity is one of the important limiting factors for the clinical use of this drug, the exact mechanism underlying the cardiotoxicity is still under debate and different experimental protocols were used. Lipidomics technology was used in this study to investigate the underlying the cardiotoxicity is still under debate and different experimental protocols were used. Lipidomics technology was used in this study to investigate the underlying mechanism of cardiotoxicity induced by DOX. Lipidomics refers to the complete analysis of lipid profile of a cell or organism based on the principles and tools of analytical chemistry particularly mass spectrometry. This study was designed to investigate cardiotoxicity induced by doxorubicin using lipidomics technology.

Method: Twelve adult male rats divided randomly into two groups, each group comprising of six rats. 1: Control group (single dose (1ml) saline intraperitoneally); 2: DOX group (20 mg/ kg single dose intraperitoneally). After anesthesia, the myocardial tissue harvested and stored in liquid nitrogen, then the metabolites will be extracted from left ventricle of the heart tissue, derivatized using boron trifluride-methanol 10% and then the metabolites identified using GC-MS.

Results: The results showed that treatment with DOX produced significant (P<0.05) increase in the level of acetic acid, cholesterol, myristic acid, and stearic acid. Whereas the level of arachidonic acid, linolic acid, pentadecanoic acid, oleic acid and ricinoleic acid, decreased significantly (P<0.05) in DOX group. Lauric acid, palmitic acid, and methylcyclohexane, were found to be increased in DOX group.

Conclusion: This study showed that DOX induced cardiotoxicity can be identified by lipidomics technique by measuring lipid biomarkers of cardiotoxicity in heart tissue which include the saturated fatty acids (stearic acid, acetic acid and palmitic acid), unsaturated fatty acids (arachidonic acid, linoleic acid, and oleic acid) as well as cholesterol.

Key words: Lipidomics, doxorubicin, cardiotoxicity.

تطبيق اللبيدومكس لشرح تأثير دواء الدوكسوروبسين الحاد السام للقلب

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الخلاصة:

تأثير التسمم الحاد لعضلة القلب بواسطة دواء الدوكسوروبسين (Doxorubicin) هو احد العوامل الهامة التي تحد من الاستخدام الطبي لهذا الدواء. الميكانيكية الدقيقة المسببة لتسمم عضلة القلب لاتزال قيد التجربة وباستخدام مختلف البروتوكولات التجريبية. تم استخدام تقنية اللبيدومكس (lipidomics) في هذه الدراسة لتشخيص تقنية تسمم عضلة القلب بواسطة دواء الدوكسوروبسين. تشير اللبيدومكس الى التحليلات الكاملة للدهنيات الموجودة في الخلية او العضيات بناءا على مبادئ وادوات الكيمياء التحليلية وخاصة قياس الطيف الكتلي. صممت هذه الدراسة لتشخيص سمية عضلة القلب بدواء الدوكسوروبسين بواسطة استخدام تقنية اللبيدومكس.

طرائق العمل:

تم تقسيم أثنا عشر جرذ ذكر بالغ بصورة عشوائية الى مجموعتين ,كل منها يتكون من ستة جرذان

١.مجموعة االسيطرة والتي اعطيت جرعة واحدة من المحلول المالح داخل الصفاق (intraperitoneally).

٢.مجموعة الدوكسوروبسين والتي اعطيت (٢٠ ملغ/كغ جرعة واحدة) داخل الصفاق (intraperitoneally).

بعد تخدير الجرذان تم استئصال نسيج القلب وحفظه في النيتروجين السائل ومن ثم المتئيضات تم استخلاصها من البطين الايسر لنسيج القلب و تم اشتقاقها باستخدام boron trifluride مع الميثانول بتركيز ١٠٪ ومن ثم تم تشخيصها باستخدام جهاز ال. GC-MS

النتائج:

أظهرت النتائج ان المعالجة بدواء الدوكسوروبسين أظهرت زيادة بشكل ملحوظ ($P<0.05$) في مستوى حمض الاستيك (الخليك)، حمض المارستك، حمض الستياريك والكوليسترول، بينما وجد ان هناك انخفاض بشكل ملحوظ ($P<0.05$) في مستوى حمض الأراكيدونيك، حمض اللينولييك، حمض الاوليك، حمض الرسن اولك وحمض البنتاديكانوك في مجموعة الدوكسوروبسين، ووجد كذلك ان مستوى حمض اللورك، حمض البالمتيك والميثيل سيكلوهكسان ازداد في مجموعة الدوكسوروبسين.

الاستنتاج:

اظهرت هذه الدراسة ان تسمم عضلة القلب الناتج من دواء الدوكسوروبسين تم تشخيصه عن طريق استخدام تقنية اللبيدومكس عن طريق قياس المؤشرات الحيوية لدهنيات نسيج عضلة القلب والمقترنة بتسممه والمتضمنة الاحماض الدهنيه المشبعة (حمض الستيارك، حمض الاستيك، وحمض البالمتيك)، الاحماض الدهنية غير المشبعة (حمض الأراكيدونيك، حمض اللينولييك، وحمض الاوليك) وكذلك الكوليستيرول.

لكلمات المفتاحية: اللبيدومكس، الدوكسوروبسين، تسمم عضلة القلب.

Introduction

Medical researchers usually concerned with drugs toxic effects in the evaluation of toxicity and the secondary drug development. Acute cardiotoxicity occur as result of adverse reaction of drugs is very high and often the recovery is difficult. Serious cardiotoxicity caused by many drugs, might be manifested as heart failure (HF) and elevated blood pressure which may lead to death. There are many drugs like doxorubicin (DOX), which is potent anticancer drug belong to anthracycline family, have limited use because of its serious cardiotoxicity[1]. The definition of cardiotoxicity is the appearance of dysfunction in cardiac muscle as a result of exposure to anticancer therapy and it depends on the kind of anticancer therapy, and on the system of detection used[2]. Unfortunately, DOX has strong toxicity on noncancerous tissues like, the heart, kidneys, etc. However, it is well recognized that the most sever toxic effect of DOX is on the heart tissue[3]. Cardiotoxicity can be acute or chronic, permanent or transient, and can affect the contractility of the myocardium, the conduction of cardio-myocyte or the vascular system of myocardium[4]. Lipidomics is end point research which deals with metabolic lipid profile of the cell. They change continuously since they are affected by various states, not just due to the lipids

consumed as part of the diet, but also through the underlining disease, and many other factors [5].

Changes that occur in lipid structure, expression and function can induce diseases like metabolic disorders, cardiovascular disease and cancer. Now, lipidomics enable the study of the expression and localization of the whole lipid profile. lipidomics, as part of the "omics" field of researches, offers understanding of the pathways by which lipids function as a part of the biological system. Lipidomics can identify disease biomarkers that are previously unknown, improve lipid-related disease diagnosis, and develop novel pharmacological therapeutics, further opening the door to personalized medicine[6] .The cardiotoxicity induced by doxorubicin can be identified by measuring the level of fatty acids of the heart tissue using gas chromatography mass spectrometry.

Aim of study

This study aimed to evaluate the cardiotoxic effect of DOX to myocardium through the study of lipid profile of the heart tissue (lipidomics study).

Materials and Methods

Animals

Twelve adults male Wistar rats were used in the study purchased from animal house - college of Pharmacy-Mustansiryiah university. The rats were kept in cages with free access to food and water. The cages were placed in a quiet and temperature-controlled room in which a 12:12-hour light-dark cycle was maintained. The weight of the rats varied between 200-220gm. The rabbits were allowed a ten days acclimatization period before being used in experiments.

Study design

The rats divided randomly into two groups, each consisting of six rats. The Control group received single dose of saline injection at a dose of 1 ml via intraperitoneal route. The DOX group received (20 mg/ kg single dose) intraperitoneally.

Induction of cardiotoxicity

Induction of cardiotoxicity carried out by the administration of DOX intraperitoneally in a dose of 20 mg /kg as a single dose for acute cardiotoxicity induction[7] .

Sample collection and preparation

At the end of the experiment, the rats were anesthetized by intramuscular administration of 50 mg/kg ketamine and 5 mg/kg xylazine[8]. The heart is immediately removed and washed with tap water then distilled water and rapidly stored in liquid nitrogen.

Cardiac lipidomics

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[9] . 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 by acid catalyzed esterification(9)**:**Briefly, 150 µL chloroform: methanol (1:1) and 100 µL of 10% BF3 in methanol was added to each of the stored dried extracted samples of the upper layer of the heart tissue. Samples were vortexed for one minute and heated for 90 minutes at 80 ºC. Once samples had cooled, 0.6 ml hexane and 0.3 ml water (HPLC grade) was added. After vortexing for one minute, the organic layer

(upper layer) was transferred to glass screw cap tube and left to dry overnight. Samples were reconstituted with 0.5 mL hexane and 3 µL from each sample were injected into a GC-MS.

Aquadruple GC-MS analysis condition[10]**:** 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 was 0.32 mm I.D. and 0.25 µm.

The GC-MS operation procedure: The GC oven heated as 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 occur according to Fiehn and Kind, 2005[11,12] 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. 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: Myocardium tissue metabolomics particularly lipidomics showed total fatty acids, and other metabolites, distributed between control and DOX treated group as shown in fig. (1). Table (1) showed that acetic acid, cholesterol, myristic acid, stearic acid found to be increased significantly ($P<0.05$) in DOX treated group. Arachidonic acid, linolic acid, pentadecanoic acid, oleic acid and ricinoleic acid, decreased significantly ($P<0.05$) in DOX group. Lauric acid, palmitic acid, and methylcyclohexane, were found to be increased in DOX group, whereas tridecanoic acid, cyclohexane, hydroxylamine, methylcyclopentane, and 3-methylhexane were decreased in DOX treated group. Dichloroacetic acid, erucyl amide, margaric acid, octadecadienoate, octadecenyl aldehyde, palmitaldehyde, phthalic acid, and undecanoic acid found only in control group and absent from DOX group whereas brassidic acid found only in DOX treated group. Metabolomics amount were measured in relative levels to control group as showed in fig. (2).

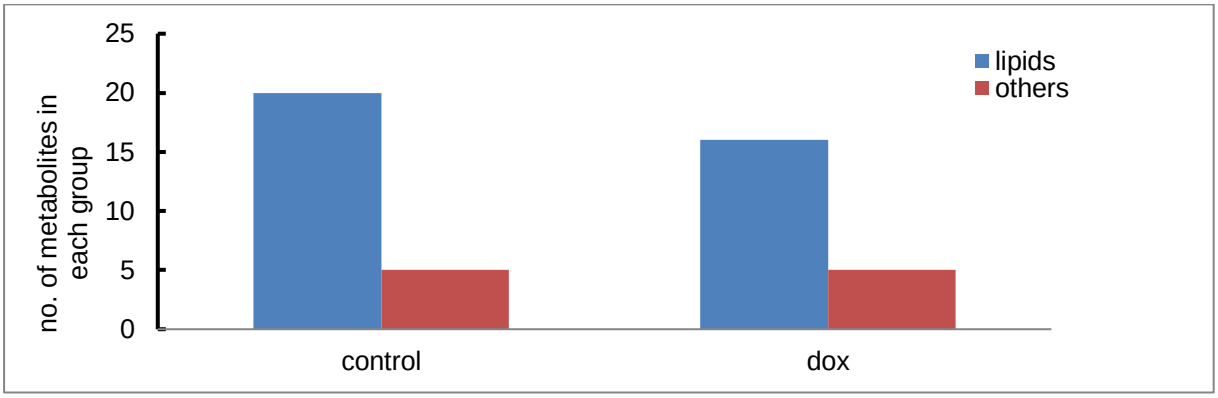

Figure (1): Number of metabolites in control and DOX treated groups

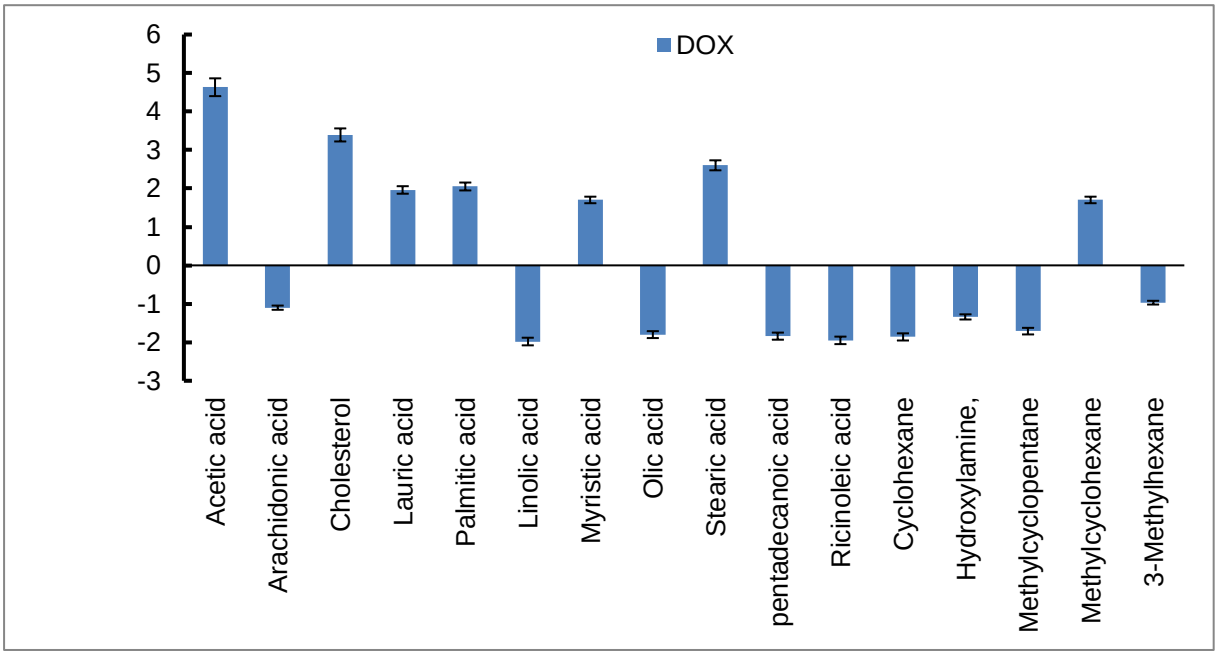

Figure (2): Fold change in myocardial tissue metabolomics after treatment with DOX treated groups data present in log relative levels to control

Table-1: Cardiac metabolomics of control and DOX treated groups (heart tissue derivatized BF3-methanol 1%).

Metabolom	Control	DOX
Acetic acid	0.1767±0.04	74.99±2.01*
Arachidonic acid	4.992±0.16	0.02±0.00*
Brassidic acid	–	0.19
Cholesterol	0.1967±0.10	5.08±0.23*
Dichloroacetic acid	0.59	–
Lauric acid	0.7483±0.24	1.435±0.41
Erucyl amide	4.57	–
Palmitic acid	1.905±1.38	4.05±1.10
Linolic acid	2.308±0.76	0.125±0.02*
Margaric acid	15.09	–
Myristic acid	1.348±0.10	2.03±0.21*
Oleic acid	0.6717±0.16	0.245±0.10*
Stearic acid	1.187±0.52	5.9±0.27*
Octadecadienoate	7.33	–
Octadecenyl aldehyde	1.05	–
Palmitaldehyde	4.52	–
Pentadecanoic acid	0.51±0.02	0.16±0.02*
Phthalic acid	0.59	–
Ricinoleic acid	4.96±0.30	0.55±0.05*
Tridecanoic acid	0.1867±0.01	0.185±0.03
Undecanoic acid	0.1	–
Cyclohexane	7.6	2.04
Hydroxylamine	0.78	0.61
Methylcyclopentane	25.76	12.71
Methylcyclohexane	0.505	0.76
3-Methylhexane	2.36	2.14

high conc.	low conc.

The data presented as Mean ± SE, *: $p<0.05$ compare to control

Discussion

In this study, metabolomics analysis based on GC-MS was used to display the lipidomics profile of myocardium with the acute toxicity induced by DOX. DOX, is one of Anthracycline antibiotic family, recognized as one of the widely used and most effective chemotherapeutic agent for treatment of several types of human cancers. However, the clinical use of DOX is limited because of its significant cardiotoxicity, which often resulting in the progression of permanent degenerative cardiomyopathy and eventually heart failure[13].

Lipidomics can play an important role in mechanistic studies, prediction of risk factors, and therapeutic monitoring for metabolic diseases given the strong association of lipids with these diseases like cardiac problems[14]. Lipidomics application in vascular health research, toxic and ischemic heart diseases has established utilities in population profiling, identification of biomarker, pathogenesis studies, and monitoring the responses to

therapy through the systematic quantitative analysis of multiple classes of lipids including oxidized lipids[15]. The myocardium is the greatest energetically demanding organ in the body, and mainly utilizes long-chain fatty acids and glucose as the main substrates to generate ATP, that is required for the contractility of myocardium[4,16].

An important finding is that the changes in fatty acid levels were very different, where there were upgrading in the level of the saturated fatty acids while there were downgrading in the unsaturated fatty acids level. The level of saturated fatty acids (stearic acid, palmitic acid, myristic acid, lauric acid, and acetic acid) found to be increased significantly (P<0.05) by nearly two folds elevation in DOX treated rats (table-1) , this increase may indicate that there was inhibition in the ß-oxidation of the saturated fatty acids. It is known that fatty acids ß-oxidation is tightly linked with glycometabolism in the heart. The main cause of reduction in fatty acids oxidation was inhibition of TAC cycle (citrate cycle) and oxidative phosphorylation which occur as a result of DOX accumulation in the heart. Therefore, the acyl-CoA and NADH accumulation should have occurred in DOX-treated myocardial cells. Down-regulation of the two enzymes, NADH dehydrogenase (NADHD) and carnitine palmitoyltransferase I (CPTI) in DOX treated myocardial cells were found in the previous studies, which gives indirect evidence to the assumption[17] .

However, the level of unsaturated fatty acids (oleic acid, linoleic acid, arachidonic acid, and ricinoleic acid) were found to be decreased significantly (P <0.05) (table-1) by approximately two folds (fig. 2) in DOX treated group. One of the possible explanation was that the unsaturated fatty acids undergo peroxidation in the presence of DOX (a process by which oxidants like free radicals can attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids[18]. The abnormal oxidation process of fatty acids responsible for excessive oxidation damage on the mitochondria of the myocardium.

The normally beating heart required high energy supply which is largely depend on the supply of fatty-acyl coenzyme A[19] . In the present study the level of acetate increased significantly (P <0.05) nearly by two-fold change(fig.2) in DOX treated group. Acetate considered as a vital source of acetyl Coenzyme A (acetyl-CoA) and it played an important role in maintaining the homeostasis of energy in mammalian cells[20]. Acetyl-CoA is mainly used in anabolic processes like the biosynthesis of fatty acids and in catabolic process (TCA cycle) as an energy generator. In the mitochondria, acetyl-CoA derived from pyruvate and acetate, enters into the TCA cycle in order to meet energy requirements[21]. DOX reduce the uptake of acetate by the cells, this may illustrate the reduced ability of the mitochondria to metabolize the acetate to produce energy. Several previous studies showed that the pyruvate transport rate was inhibited by DOX. In this context, Andreadou et al. 2009 made in vivo metabolomics study using NMR spectroscopy to prove significantly increased acetate levels in myocardium tissue exposed to DOX, the authors suggested that these metabolites which produced intracellular considered as a novel biomarker for DOX cardiotoxicity[22].

The level of cholesterol was shown to be elevated significantly (P <0.05) (table-1) in DOX-induced group, increased two folds (fig. 2) when compared to control 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[17,23].

Conclusions

From this study and according to the results above, DOX lipidomics biomarkers associated with cardiotoxicity were identified and it can be concluded that DOX administration could affect myocardial function by producing several changes in

lipid metabolism which are considered as a primary source of energy for this organ. Whereas the unsaturated fatty acids undergo peroxidation particularly arachidonic acid, linoleic acid, and oleic acid as a result of increased oxidative stress produced by DOX, so these fatty acids expected to be biomarkers of cardiotoxicity induced by DOX. Also, acetic acid might be considered as a biomarker of cardiotoxicity since DOX reduce the uptake of acetate by the cells, as a result, the ability of the mitochondria reduced to metabolize acetate to produce energy. The abnormal oxidation process of all these fatty acids responsible for excessive oxidation damage on the mitochondria of the myocardium leading to heart failure. Cholesterol also accumulated in DOX treated hearts which could be considered a characteristic of cardiotoxicity induced by DOX.

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