10.21608/avmj.2025.314511.1365 Assiut University web-site: www.aun.edu.eg

CARDIOPROTECTIVE INFLUENCE OF METFORMIN ON DOXORUBICIN-INDUCED CARDIOMYOPATHY: THE IMPLICATION OF MITOCHONDRIAL DYNAMICS

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Received: 1 September 2024; **Accepted:** 28 October 2024

ABSTRACT

Doxorubicin (DOX) is a potent cytotoxic drug for treating various types of tumors. However, the limitation of its use is due to its potential for cumulative and dose-dependent cardiotoxicity. Recently, it has been suggested that the accumulation of damaged mitochondria, through dysregulation of the mitochondrial fission/fusion dynamic process, may be a critical mechanism contributing to DOX-induced cardiotoxicity. Metformin (Met), an antihyperglycemic drug, has been reported to exert cardio protection against DOX-induced Cardiotoxicity. We supposed that metformin medication could protect the heart from DOX induced cardiotoxicity via modulating mitochondrial dynamics. 32 adult male rats (190–210 g) were haphazardly separated into four groups. Control group was taken 1 ml of normal saline orally every day; DOX group: taken 3 mg/kg twice a week; Met group taken metformin (250 mg/kg/day) orally; and DOX+ Met group: received Met and DOX. After two weeks, the heart muscle of each rat was extracted, and each left ventricle was dissected out and cut into two parts. The primary part was placed in formic aldehyde for histology examinations. The other part was stored at −80°C for measurements of proteins of mitochondrial fusion expression, mitofusin 1 and mitofusin 2 (MFN1 and MFN2), which were estimated by real-time PCR. The results admit that Met medication affected the interpretation of MNF1 and MFN2, which significantly increased in the DOX+ Met group, compared to the DOX group. In conclusion, we discovered that metformin treatment could improve the mitochondrial dysregulation created by DOX.

Keywords: Doxorubicin, Cardiotoxicity, Metformin, MFN1, MFN2

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INTRODUCTION

Doxorubicin (DOX) is a cancer treatment that is intended for medicine for a lot of cancer types, such as leukemia, malignant lymphoma, and other types of cancer (Dundar *et al.,* 2016). Although its efficacy is like other anthracyclines, DOX has severe side effects in heart tissue. DOX causes aggregated and dose-dependent heart damage, extending from cardiomyocyte dysfunction to serious cardiotoxicity, which finally leads to cardiomyopathy and heart failure (Zagar *et al.,* 2016).

There are multiple proposed mechanisms for doxorubicin-induced cardiotoxicity (DIC). The most significant way is the reactive oxygen species (ROS) formation, which causes oxidative stress (Nguyen *et al.,* 2023). Mitochondria are subcellular organelles that occupy around 40% of each cardiomyocyte volume, making them the major source for ROS endorsing (Osataphan *et al.,* 2020). In addition, mitochondria are considered the most organelle responsible for DOX-induced cardiotoxicity. Therefore, preservation of mitochondrial balance is critical to keep cardiac cells in a healthy condition during DOX therapy (Iborra-per niche *et al.,* 2024).

Mitochondrial dynamic is the system in which mitochondria get concerted energetic cycles. This system of mitochondria is closely associated with mitochondrial function to keep energy metabolism, intracellular calcium balance, and cell surveillance (Ren *et al.,* 2020). The mitochondrial proteins responsible for fusion are mitofusin 1 (MFN1), mitofusin 2 (MFN2), which are present in the outer mitochondrial membrane. Also, the optic atrophy 1 (OPA1) protein is present in the inner mitochondrial membrane. On the contrary, mitochondrial proteins responsible for fission, mitochondrial fission protein 1 (Fis1) and dynamin-related protein 1 (Drp1), which cleave mitochondria (Zerihun *et al.,* 2023). accumulation of malfunction mitochondria through a change in energetic system has been observed as a possible factor in causing DOX heart damage lately. Extreme mitochondrial fission with impaired fusion that occurred by DOX induction could critically raise mitochondrial fragmentation and cardiotoxicity. Even so, this can be reversed by using either inhibitors of fission proteins or fusion promoters, which can provide more protection to the heart against DOX-induced heart damage.

Metformin (Met) is an anti-diabetic treatment that acts primarily as a medicine in hyperglycemia care (Baker *et al.,* 2021). Previous studies have observed that Met has a protective influence on heart tissue during DOX widely, through inhibition of oxidative damage and cardiotoxicity (Bu *et al.,* 2022). However, the mechanisms by which Met affects cardiac mitochondrial function and mitochondrial energetic balance are still not entirely understood. In the present study, we examined Met's cardioprotective influence on DIC. We hypothesized that Met could exert cardioprotective effects via the promotion of mitochondrial fusion.

MATERIALS AND METHODS

1. Experimental animals:

The study was done on 32 male albino rats aged about 6-8 weeks and with body weights (190 -210 grams). Rats were obtained from the Animal House of the Faculty of Medicine at Assiut University and housed in metal cages under healthy conditions (temperature 22°C, humidity 50%), according to the classification of groups. They were maintained on natural light and dark cycles. The rats had free access to food and water. Animal care and treatment adhered to the guidelines set by the Animal House of Assiut University, following ethical standards for the use of experimental animals.

2. Experimental design:

Animals were randomly assigned to four groups (n = 8 each), with dosing and treatment durations designed according to the protocol outlined by **Chen *et al.* (2020).**

- **Group I (control group):** rats were given normal saline (1 ml/kg) orally every day for 14 days.
- **Group II (treated with DOX):** Rats received intraperitoneal (IP) injections by DOX every other day for 14 days at a dose of 3 mg/kg.
- **Group III (treated with Met):** Rats were administered metformin at a dosage of 250 mg/kg/day through gastric gavage for 14 days.
- **Group IV (treated with DOX and Met):** rats received metformin (250 mg/kg/day) via gastric gavage for 14 days, and DOX IP (3 mg/kg) every other day for 14 days. Doxorubicin Hydrochloride (DOX·HCl) and metformin were purchased from Bio-Technology Co., Ltd. (Shanghai, China) and in group IV Quanao Chemical Co., Ltd. (China), respectively.

3. Collection of Heart Specimens.

After the treatments were completed, the animals were weighed and then sacrificed. The heart tissues were removed and weighed. For histopathological examination and immunohistochemical enzymatic analysis, a portion of each heart tissue was placed in 10% formalin. for the biochemical analysis, another portion was kept at -80°C and thawed just before being homogenized in phosphate-buffered saline.

4. Real-time qPCR:

Real-time qPCR was used to detect relative gene expressions of mitofusin (MFN1, MFN2) in LV tissue. RNeasy Mini Kit (Catalog no. 74104, Qiagen, Germany) was used to isolate RNA from frozen tissue. After quantification using a nanodrop spectrophotometer, aliquots of RNA were reverse transcribed using a reverse transcription kit. (catalog no. 4374966, Thermo-Fischer Scientific, USA). Maxima SYBR Green qPCR Master Mix kit, (Catalog no. #K0251, Thermo-Fischer Scientific, USA) was used to amplify MFN1, MFN2, and β-actin (reference gene) genes. The amplification was performed using the primer sequence described in Table 1. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2023).

Table 1: The sequences of the PCR primers

Gene	5′-3′ primer sequence
MFN1	Forward: ATGAGATTTGTCGCCTGTC Reverse: GGGATTACTGATGAACCG AAG
MFN2	Forward: TTGTGGAGTATGC Reverse: ATGCCATCTTCTAT
β- actin	Forward: CACTATCGGCAATGAGCG GTTCC Reverse: CAGCACTGTGTTGGCATA GAGGTC

5. Histopathological evaluation

Heart tissue samples were preserved in 10% buffered neutral formalin for 48 hours. Following standard tissue processing, which involved dehydration with alcohol and clearing with xylene, the samples were embedded in paraffin. The paraffin blocks were sliced into 5 µm sections, then deparaffinized in xylene and rehydrated using decreasing alcohol concentrations. After that, the sections were stained with hematoxylin and eosin (H&E) to assess various morphological changes. Finally, the findings were categorized as either positive or negative.

6. Ethics approval

This study was conducted following the principles and guidelines provided by the Animal Research Ethics Committee, Faculty of Medicine, Assiut University (26/6/2023, IRB local approval number 04-2023-300201).

7. Statistical analysis

Data were reported in terms of mean and standard deviation and analyzed using one-way analysis of variance (ANOVA),

followed by a post hoc Turkeys test using Prism Graph-Pad Software 5.03. A significance level of p <0.05 was judged statistically significant.

RESULTS

1- The metformin effect on mitochondrial fusion in rats intoxicated with DOX.

Metformin demonstrated a defending effect against DOX-induced cardiotoxicity by influencing the MFN1 and MFN2 expression levels. The results showed that the relative mRNA expressions of MFN1 and MFN2 were reduced significantly in the group treated with DOX (P < 0.001), compared to the control and Met groups. However, metformin treatment significantly increased the expressions of MFN1 and MFN2 (p < 0.05, p < 0.01) respectively, compared to DOX-treated group (Fig. 1).

Table 2: Effect of metformin on mitochondrial fusion proteins MFN1 and MFN2 relative mRNA expression in DOX- intoxicated rats

	Control (N=8)	DOX (N=8)	Met (N=8)	DOX and Met (N=8)
MFN1 (Fold change)	0.995±0.052	0.48±0.128 a***	1.003±0.120 **b*****	0.638±0.113 a***b*c***
MFN2 (Fold change)	0.995±0.05	0.33±0.088 **a*****	1.005±0.119 **b*****	0.556±0.141 a***b**c***

Data presented as mean ± SD. One-way ANOVA followed by a post hoc Turkeys test were used to perform the statistical analyses, a. **a** statistically significant difference from the control group value. **b** statistically significant from the Dox-treated group value. c statistically significant from the Met-treated group value. *** mean ($p \leq 0.001$), ** mean ($p \leq 0.01$), and * mean ($p \leq 0.05$). Met: metformin, DOX: doxorubicin; DOX and Met: doxorubicin plus metformin.

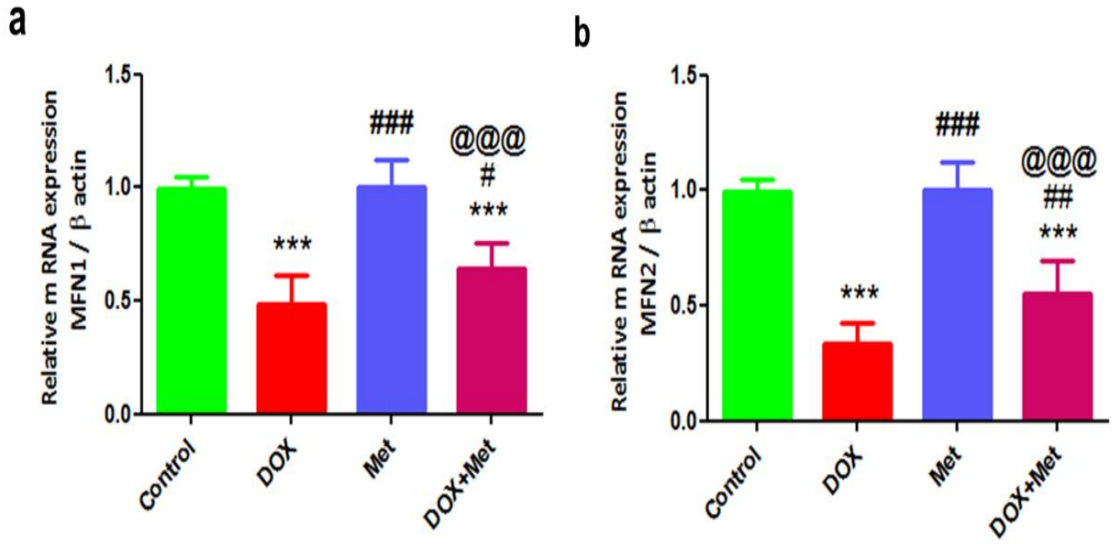

Figure (1): The effects of metformin on MFN1 and MFN2 expression in the DOX- intoxicated rats by real-time qPCR analysis. β-actin was utilized to normalize expression data. Data were expressed as means ± S.D. ***p <0.001 vs control group, $^{\#}$p <0.05, $^{\#\#}$p <0.01, $^{\#\#\#}$p <0.001 vs DOX group, $^{@@@}$ p <0.01 vs Met group.

2. Histological Results

Light microscopic examination of the myocardium of the left ventricle stained with H&E In group I, the myocardium showed normal architectural features of cylindrical irregular branched muscle fibers with central oval vesicular nuclei and acidophilic sarcoplasm. Narrow spaces of endomysium

were present between the muscle fibers (Fig.2a). In Group II (DOX-treated group), the myocardium showed marked deterioration within the myocardial fibers with vacuolar changes within the sarcoplasm of some myocardial cells, especially around nuclei, loss of some myocardial striations, and many deeply stained pyknotic nuclei. Wide interfiber spaces between the myocardial fibers, and focal areas of deeply acidophilic sarcoplasm of degenerated myocardial fibers were also noticed (Fig.2b). While in Group III (Met treated group), the myocardium showed more or less normal configuration and architecture of branched myocardial fibers with central oval vesicular nuclei, acidophilic sarcoplasm, and narrow clear endomysium (Fig.2c). In group IV (DOX and Met-treated group), some myocardial fibers showed loss of myocardial striations with focal vacuolar changes of sarcoplasm with few pyknotic degenerated nuclei. A few inflammatory foci, which are characterized by cellular infiltrations, were revealed between some fibers (Fig. 2d).

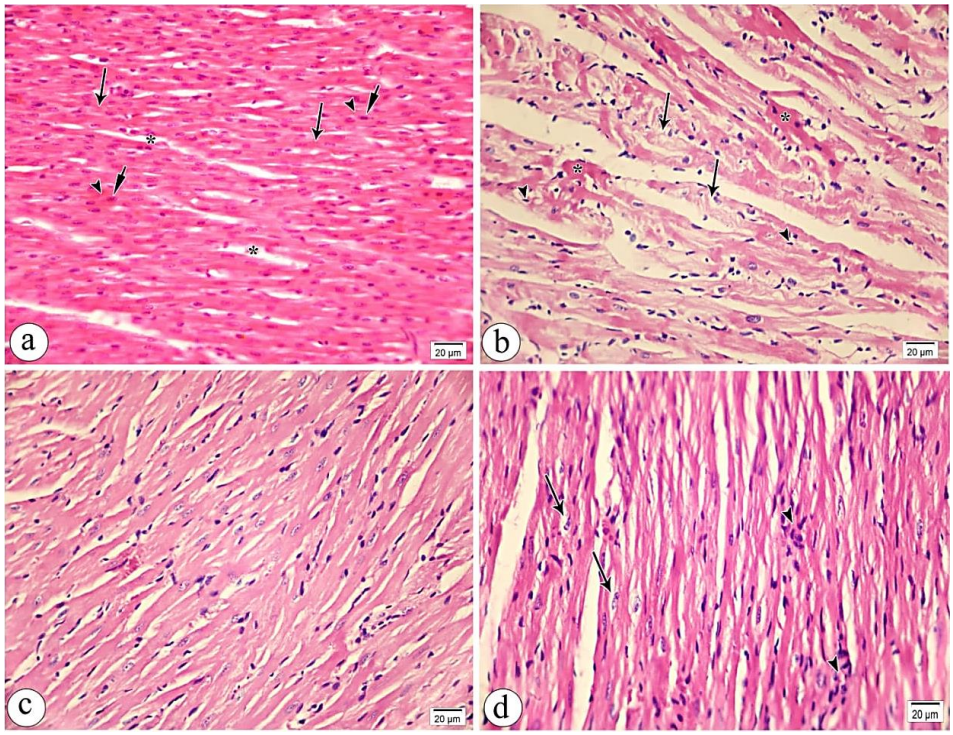

Figure (2): A photomicrograph of myocardium of left ventricle stained with hematoxylin and eosin (H&E) at x400 magnification. a. GI (Control Group) showing normal architectural features of the myocardium, muscle fibers were cylindrical and irregularly branched (arrows) with vesicular central nuclei (short arrows), and sarcoplasm appears acidophilic (arrowheads). Notice narrow spaces of endomysium in between the muscle fibers (*) are present. b. GII (diseased group) shows marked deterioration within the myocardial fibers with pale vacuolar sarcoplasm of many fibers (arrows) and small dense nuclei (arrowheads). Notice focal areas of deeply acidophilic sarcoplasm are present (*) of degenerated myocardial fibers. c. G III (metformin-treated group) showed no apparent pathological changes in myocardial fibers with more or less normal configuration and architecture. d. G IV shows focal vacuolar changes of sarcoplasm of few myocardial fibers (arrows) with few inflammatory foci, which are characterized by cellular infiltrations (arrow heads).

DISCUSSION

Recent research has shown that metformin has multiple effects, stemming from its ability to lower blood sugar levels. These include suppression of tumor growth, extension of lifespan, and a reduction in cardiovascular disease incidence in animal models (Galal *et al.,* 2024). DOX exhibits a wide range of anti-cancer properties and is

commonly utilized in clinical settings to enhance the outcomes for cancer patients. (Mujwar *et al.,* 2023). However, it causes cumulative and dose-dependent cardio-toxicity, which limits its clinical use. DOX acts via multiple mechanisms of action associated with cardiotoxicity. Mitochondria are often regarded as the primary target of DOX, with mitochondrial dysfunction being a key characteristic of DOX-related cardiotoxicity (Wu *et al.,* 2022).

Cardiomyocytes, which can contain as many as 6,000 mitochondria and make up 30% to 40% of the cell's volume, are among the cell types with the highest ATP consumption. Because cardiomyocytes depend heavily on power generation, they are especially vulnerable to damaging materials that cause mitochondrial dysfunction (Cheung *et al.,* 2019).

Doxorubicin is cationic and features both hydrophilic and hydrophobic regions, enabling it to easily pass through the membranes of cytoplasmic organelles. Reports indicated that DOX accumulates in mitochondria at concentrations 100 times greater than those found in plasma. (Marais, 2014). By accumulating in the mitochondria of cardiomyocytes, DOX increases the production of reactive oxygen species (ROS) while reducing energy production, leading to cell apoptosis. An overload of intracellular ROS is linked to changes in mitochondrial cycles, which are crucial for preserving mitochondrial function and facilitating mitophagy (Vitale *et al.,* 2024).

Fusion and fission dynamics of mitochondria are critical regulators of cardiac function. Abnormalities in these dynamics, marked by increased mitochondrial fission and reduced fusion, are significant contributors to DOX-induced myocardial injury in cardiomyocytes. This disruption leads to morphological abnormalities and dysfunction of mitochondria, resulting in cardiomyocyte dysfunction and then apoptosis (Wang *et al.,* 2024, Huang *et al.,* 2022).

Alongside mitochondrial fission, mitochondrial fusion is essential for maintaining mitochondrial health. Enhancing mitochondrial fusion by upregulating fusion-related proteins can mitigate myocardial damage (Ding *et al.,* 2022; Ong *et al.,* 2022) and does not compromise mitochondrial quality in the heart under normal conditions (Qin *et al.,* 2020), making it a safer approach compared to inhibiting mitochondrial fission.

Earlier studies have shown that doxorubicin disrupts the balance of cardiac mitochondrial dynamics by inhibiting mitochondrial fusion processes, which in turn promotes apoptosis mediated by mitochondrial dysfunction in mice (Attachaipanich & Chattipakorn, 2023; Jia *et al.,* 2018; Tomczyk *et al.,* 2022). Consistent with these studies, our findings revealed a reduction in the mitochondrial fusion proteins' mRNA expression of MFN-1 and MFN-2 in DOX-intoxicated rats compared to control groups, indicating a decline in cardiac mitochondrial biogenesis.

A previous study proved that exposure to DOX resulted in the upregulation of FoxO1, a transcription factor from the Forkhead family that plays a crucial role in regulating myocardial homeostasis and negatively impacts MFN2 transcription. Additionally, overexpression of MFN2 was shown to enhance mitochondrial fusion and mitigate oxidative stress, apoptosis, and cardiac dysfunction induced by DOX (Jia *et al.,* 2018). Since DOX disrupts mitochondrial dynamics, reducing mitochondrial fission, and promoting fusion appear to be effective strategies for lessening cardiac dysfunction associated with DOX treatment.

Preventing mitochondrial fragmentation by restoring MFN1 and MFN2 offers protection against cell death and heart failure caused by DIC (Chen *et al.,* 2021). Several pharmacological interventions with antioxidant characteristics, such as metformin, have shown potential in mitigating cardiotoxicity induced by chemotherapy (Ashour *et al.,* 2012).

Metformin has been reported to provide mitochondrial protection in models of DOX-induced cardiotoxicity (Argun *et al.,* 2016). Previous studies have shown that metformin enhances mitochondrial dynamics, bioenergetics, and biogenesis by increasing fusion proteins and reducing fission proteins (Sun *et al.,* 2023, Attachaipanich & Chattipakorn, 2023).

The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is described as the master regulator of mitochondrial biogenesis and function (Shelbayeh *et al.,* 2023). A previous study reported that overexpression of PGC-1α increased expression of MFN1 and MFN2 in the hearts of mice. Conversely, hearts of PGC-1α-deficient mice resulted in a modest but significant decrease in MFN1 and MFN2 mRNA levels (Kelleni *et al.,* 2015). Interestingly, metformin has been found to efficiently induce PGC-1α expression/activity, thus affecting its downstream pathway and correcting the abnormal mitochondrial biogenesis, function, and dynamics that take place (Izzo *et al.,* 2017; Wu, 2023).

In our research, we discovered that metformin could reduce DIC by enhancing the expression of MFN1 and MFN2. Furthermore, the mitochondria, that have been separated, come together to form a long-interconnected structure, maintaining electrical and biochemical connections and repairing any damaged mitochondria. Therefore, it seems that allowing mitochondria to fuse is beneficial, and preventing this fusion can negatively impact cell function. In conclusion, the present findings reveal that treatment with metformin could attenuate DOX-induced cardiotoxicity by promoting mitochondrial fusion, which is supported by histopathological findings.

Statements & Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript."

Author Contributions

Nashwa Maghraby: conceptualization, writing – original draft, formal analysis. **Mona A.H. EL-Baz:** writing–review and editing, supervision. **Athar M.A. Hassan:** investigation, methodology, validation**. Sary Kh. Abd-Elghaffar:** supervision, reviewing, and editing**. Amira S. Ahmed:** methodology, formal analysis, writing the original draft**. Mahmoud S. Sabra:** methodology, formal analysis, writing the original draft

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التأثير القلبي للميتفورمين على تسمم القلب الناجم عن الدوكسوروبيسين: الآثار المترتبة على ديناميكيات الميتوكوندريا

نشوى عبد الغفار مغربي ، منى عبد الحميد الباز،آثار محمود عبد الرحمن ، ساري خليل عبد الغفار ، أميرة سالم احمد ، محمود سيد صبره

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يعد عقار دوكسوروبيسين من العوامل الكيميائية العلاجية القوية لعلاج الأورام الخبيثة الصلبة والدموية المتعددة, ويسبب سمية قلبية تراكمية تعتمد على الجرعة، مما يحد من استخدامه. ومؤخرًا، تم تحديد تراكم الميتوكوندريا غير الوظيفية عن طريق تغيير عمليات الانشطار/الاندماج الديناميكية للميتوكوندريا كآلية محتملة وراء السمية القلبية الناجمة عن عقار دوكسوروبيسين. عقار الميتفورمين وهو عقار مضاد لفرط سكر الدم، يمارس حماية للقلب ضد السمية القلبية الناجمة عن عقار دوكسوروبيسين. افترضنا أن العلاج بالميتفورمين يمكن أن يوفر حماية للقلب ضد السمية القلبية الناجمة عن عقار دوكسوروبيسين من خلال تعديل ديناميكيات الميتوكوندريا. وقد تم تقسيم إجمالي ٣٢ فأرًا ذكرًا بالغًا (١٩٠-٢١٠ جم) عشوائيًا إلى أربع مجموعات. مجموعة سلبية: تلقت ١ مل من المحلول الملحي الطبيعي عن طريق الفم كل يوم، تلقت مجموعة DOX ٣ مجم/كجم مرتين في الأسبوع ، مجموعة :Met تلقت الميتفورمين (٢٥٠ مجم / كجم / يوم) عن طريق الفم، ومجموعة :DOX + Met تلقت Met و DOX . في نهاية فترة العلاج (١٤ يومًا)، تم استخراج قلب كل فأر وتم تشريح البطين الأيسر وتقطيعه إلى قسمين. تم وضع الجزء الأول في الفورمالين للفحوصات النسيجية المرضية، وتم تخزين الجزء الثاني عند -٨٠ درجة مئوية لقياس التعبير عن بروتين الاندماج الميتوكوندريا، (ميتوفوسين ١ وميتوفوسين ٢ MFN1) و (MFN2 والذي تم تقديره بواسطة تفاعل البوليميراز المتسلسل الوقتي). كشفت النتائج أن علاج Met يؤثر على تعبير MNF1 و MFN2 والذي زاد بشكل ملحوظ في مجموعة DOX + Met بالمقارنة مع مجموعة DOX (P<0.05) ، (P<0.01) على التوالي. وفي الختام، وجدنا أن الضرر القلبي للميتوكوندريا الناجم عن DOX يمكن تخفيفه عن طريق علاج الميتفورمين الذي يستهدف البروتينات المشاركة في اندماج الميتوكوندريا.