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Autophagy Regulation in the Context of Arsenic Trioxide-Induced Cardiotoxicity via Flaxseed Oil: Myosin Heavy Chain, BNP and SIRT1 genetic Association

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Abstract

Arsenic is extensively utilized to cure patients with acute leukemia nevertheless; its application has been limited due to its cardiotoxicity. Additionally, arsenic damaged mitochondria manifested via limiting mitochondrial membrane potential, diminishing cytochrome c level and production of mitochondrial reactive oxygen species (ROS). Furthermore, silent information regulator of transcription (SIRT1) as an autophagy biomarker was widely implicated in numerous indispensable pathways of cardiovascular diseases. Therefore, the aim of the present study was designed to investigate the cardioprotective effect and the underlying mechanism of flaxseed oil (FLX), vitamin C and their combination versus arsenic trioxide toxicity via crosslinking myosin heavy chain (α-MHC, β-MHC), brain natriuretic peptide (BNP) and autophagy biomarker SIRT1signaling pathway.

Male Wistar albino rats were administrated arsenic trioxide (3 mg/kg) for one month. Consequently animals were co-treated via FLX (1ml/kg), vitamin C. (200 mg/kg) in addition to their combination for one month. Further, Aspartate Aminotransferase (AST), C- reactive protein (CRP) and malondialdehyde (MDA) biochemical analyses were assessed. Molecular analysis for α-MHC, β-MHC, BNP and SIRT1 gene expression were also investigated.

Arsenic myocardial injury recorded a significant increment in AST, CRP as well as MDA levels that were further modulated upon co-treatment. RT-PCR Results declared a significant reduction in both α-MHC and SIRT1gene expression upon arsenic toxicity. Nevertheless, a significant up-regulation appeared in co-treated groups. On the other hand, a significant elevation in both β-MHC and BNP was reported. Meanwhile, a significant down regulation was observed post the co-treatment. In conclusion, FLX could be a promising therapeutic regimen against myocardial injury. In addition, its prospective role could be enhanced by combination with V.C.

Keywords: *Arsenic trioxide, Flaxseed oil, Cardiotoxicity, α-MHC, β-MHC, BNP, SIRT1*

1. Introduction

Arsenic is a naturally occurring heavy metal that exist in various forms in the environment [1] and in further its contamination is considered as major public health issue that it is commonly used in agriculture and industry in addition to domestic as well as technological applications. Although arsenic is included in the composition of leukemia chemical drug, it associated with dysfunction of different organs [2]. Human populations exposed to arsenic were related to different environmental diseases [1]. Exposure to arsenic is associated with various cardio-pathologic effects including ischemia, arrhythmia and heart failure. Possible mechanisms of arsenic cardiotoxicity include oxidative stress, DNA fragmentation, apoptosis and functional changes of ion channels [3].

Arsenic-induced cardiac dysfunctions have been associated to some inflammatory signaling pathways [4, 5]. Furthermore, arsenic exposure is enhanced via the environmental conditions together with individuals those are exhibiting different arsenic metabolism modality [6]. An earlier study found that cardiac tissues exposed to arsenic trioxide had a significant higher levels of oxidative stress indicators such malondialdehyde (MDA) and serum aspartate aminotransferase (AST) activity [7, 8].

C-reactive protein (CRP) is a member of proteins which was found in blood stream, its value rise in inflammation [9]. Physiologically, CRP played an important role in innate immune system that is activated in response to pathogens. CRP is created in liver as a response to inflammatory factors such as IL-6 [9]. Elevation in serum CRP value is the most

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accurate and attainable clinical indicators for inflammations and is a diagnostic biomarker for cardiac disease [10-13].

The myosin heavy chain isoforms are responsible for regulation of cardiac muscle contraction. Heart's myosin protein texture involves β-MHC with the residual bothersome α-/ β -MHC ratio leading to equalization in cardiomyocytes contractility. Exposure to oxidative stress has been shown to shift α-MHC to β-MHC that further leads to heart failure [14]. Moreover, numerous studies investigated mutations in heavy myosin chain genes that were associated with hypertrophic and dilated cardiomyopathy [15]. Previous investigation suggested that mutations in both α-MHC and β-MHC may lead to myocardial dysfunction and finally to heart failure [16, 17].

Homeostasis of cardiovascular functions arises from crosstalk of several regulatory factors. Among these is the brain natriuretic peptide (BNP), the most biochemical marker of cardiotoxicity. Different previous studies reported a significant elevation in BNP associated with cardiac dysfunction as well as heart failure. Moreover, testing BNP levels have been integrated and accepted as diagnostic approach for heart failure [18-20]. It has been previously suggest that BNP could be an important detectable biomarker of subclinical as well as clinical cardiotoxicity post high doses of chemotherapy
[21].

Autophagy is an essential cellular pathway for recycling and decaying long-lived organelles and proteins that are deposed in bi-membranous vesicles called autophagosomes. It fuses with the lysosome forming autolysosome and then it degraded and recycled. Autophagy has been reported to play a remarkable role in cardiac homeostasis. Furthermore, the disrupt of autophagy pathways could accelerate heart failure [22].

Sirtuins family or silent information regulators of transcription (SIRT) have previously been reported as the major regulator of various metabolic activities in health as well as disease status [23]. Mammalian cardiac tissue has seven Sirtuins (SIRT1–SIRT7) that expressed [24]. SIRT1 is the nicotinamide adenine dinucleotide dependent protein deacetylases. It was reported to be beneficial for the increment of lifespan, inflammatory and cardiovascular diseases[25].

SIRT1 recorded a protective effect against cardiac diseases and declared a pivotal role in regulation of various biological metabolic activities as oxidative stress detoxification, influencing of angiogenesis, intracellular handling of calcium, influencing cell survival and induction of autophagy[26]. SIRT1 operates its function through crosstalk numerous signaling pathways as p53, Fork-head box O (FOXO), and NF-κB. All these factors are closely related to the cardiovascular disease[25].Upregulation of SIRT1was previously recorded to prevent premature cardiac hypertrophy, atherogenesis, apoptosis, and cardiac tissue fibrosis as well as cardiac dysfunction [27, 28]. However, SIRT1 suppression has been associated with valvular and septal heart defects, arterial thrombosis and different cardiovascular dysfunction [29-31].

Recently, functional foods or nutraceuticals has an important role in the mitigation of cardiotoxicity especially that induced by chemotherapeutic drugs [32]. Flax has a widespread history of conventional use both as a source of oil and fiber and is developed for commercial applications [33]. Flax seed oil (FLX) has a history of dietary usage for its potential health benefits, which include anti-tumor, antimicrobial, anti-inflammatory, laxative and anti-thermogenic effects[34]. FLX is a main source of polyunsaturated fatty acid alpha-linolenic acid (ALA) [33]. ALA is a precursor of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) and plays a vital role in the cell membrane maintain. Furthermore, Flax plant contains polyunsaturated fatty acids (PUFA) especially omega-3 fatty acids. Omega-3 was previously reported inducing protective effects against cardiovascular disease, cancer, obesity and diabetes[35].

Vitamin C (L-ascorbic acid), is a water-soluble vitamin, affects various metabolic reactions and biological process in addition to its medical relevance in treating different disease. Vitamin C was reported as a key antioxidant obtained from functional food. Furthermore, it was investigated as an antioxidant to protect the defense system against damaging reactive species[36]. Previously, vitamin C investigated a vital role in cardiotoxicity protection [37, 38].

The current study is designed to estimate the effect of FLX and vitamin C in amendment toxicity induced by arsenic.

2. **Materials** and methods

2.1. Chemicals

Arsenic tri-oxide and vitamin C was provided by Sigma-Aldrich Co (St. Louis, MO, USA). Used Kits were obtained from Biodiagnostic Company (Giza, Egypt). Kits for real time polymerase chain reaction were from Qiagene (MN, USA). All chemicals and reagents which were used in the current study were of the highest analytical grade.

2.2. Animals

Male rats of species Wister albino, weighing 180 -200 gram, were provided from the animal house, National Research Center, Egypt. Rats were housed standard conditions (5% humidity, 23 ± 5°C, 53 ± and 12 h light/dark cycle). They then allowed free access

to water and pelleted standard chow diet. The authorized ethical guidelines and rules were strictly followed in all actions involving the care and treatment of animals according to Animal Care and Use Committee of National Research Center under number (3443042022).

2.3. Experimental Design

Animals were subjected to random division into five groups (each consisting10 rats) depending on the following schedule:

Group1: Rats served as negative control, Groups from 2 to 5; Rats administrated an oral dose of arsenic trioxide (3 mg/kg body weight/d) for 30 days [39]. The second group (Group 2); Arsenic -intoxicated rats were quit untreated. The third group (Group 3); Arsenic-intoxicated rats co-treated with FLX (1ml/kg) for 1 month [40].Group 4; Arsenic- intoxicated animals co- treated with vitamin C (200 mg/kg) for 1 month [37].Group 5; Arsenic- intoxicated animals co-treated with FLX, and vitamin C (by combination of doses mg/kg) for 1 month.

2.4. Sample Preparation

All of the animal groups were sacrificed at the end of the experiment, and blood samples were obtained from each animal in each group by puncturing the sublingual vein into sterilized tubes, which were then let to stand for 15 minutes to allow clotting. Centrifugation at 4000 rpm for 15 minutes was subjected to separate the serum, which was subsequently stored at 80 °C for biochemical analyses.

2.5. Biochemical measured parameters

1.5.1 C - reactive protein (CRP)

Utilizing a third generation C-reactive protein (CRPL3) assay on Cob as C702, which has a measurement range of 0.3 to 350 mg/L and allows quantification of high CRP values, levels of CRP throughout hospitalization were estimated. The Cardiac C-reactive Protein High Sensitive (CRPHS) assay on Cobas Integra was used to assess Hs CRP at 400 plus, with a 0.1 to 20 mg/L measurement range and improved accuracy at low values [41].

1.5.2 Serum Aspartate Aminotransferases (AST) Activity

Serum Aspartate Aminotransferases (AST) activity was calculated spectrophotometrically using Biodiagnostic Company's widely available kits (Giza, Egypt). In a nutshell, L-aspartate produces L-glutamate and oxaloacetate in the absence of AST. Following that, in an alkaline media, oxaloacetate combines with 2,4-dinitrophenyl hydrazine to produce the hydrazone derivative, which can be detected at 540 nm. [42].

1.5.3 Serum Malondialdehyde

Malondialdehyde (MDA) was estimated using colorimetric kits supplied from Biodiagnostic Company (Giza, Egypt). thiobarbituric acid (TBA) reacts with MDA in acid solution to give a complex of pink color. This color could be estimated on spectrophotometer at 520 and 535 nm, using 1, 1, 3, 3-tetramethoxypropaneas a standard [43].

1.5.4 RNA extraction and quantitative real-time PCR analysis (RT-PCR)

Total RNA (30–45 mg) was extracted using RNeasymini Kit (Qiagen; USA; Cat No. 74104) from heart tissue by following the manufacturer's instructions. Gene expression of α-MHC, β-MHC, BNP and SIRT was carried out in the presence of GAPHD housekeeping gene [Sequences of both forward and reverse primers are described in Table 1]. QuantiTecht SYBR green Master Mix (Qiagen; USA; Cat No. 204243) was used for one step RT-PCR quantification. The reaction was performed by Stratagene Mx3000 P QPCR instrument (Agilent Technologies, Santa Clara, CA, USA). Briefly, in a 20µl reaction volume, 2µl of extracted total RNA were added to 1µl of 2 × one step SYBR green Master Mix and 200 ng of each primer. RT-PCR Thermal cycle was as follows: 94 ◦C for 3 min, 94 ◦C for 15 s, with annealing temperature ranging from 45–58 ◦C for 20 s "responsible for to the optimum temperature of each primer" and finally extension at 72 ◦C for 15 s for 40 cycles. The relative expression of the current amplified target genes was determined by comparative thermal cycle ($2^{-\Delta\Delta CT}$) method[44].

2.6. Statistical Analysis

Statistical analyses were carried out by Instat-3 computer program (Graph pad software Inc, San Diego, CA, USA). The SPSS 16 program's One Way Analysis of Variance (ANOVA) and Post HOC tests were used to analyze the variance within the various groups. The data was presented as means and SEM. Using Tukey's test, the level of significance was established at p 0.05.

TABLE 1

Primer No.	Name of the primer	Sequence of forward primer (5' → 3')	Sequence of reverse primer (5' → 3')
1	cardiac myosin heavy chain α (α -MHC)	5'- AAGTCCTCCCTCAAGCTCATGGC-3'	5'-ATTTTCCCGGTGGAGAGC-3'
2	cardiac myosin heavy chain β (β-MHC)	5'-GCTGTTATTGCAGCCATTG-3'	5'-TTCCTGTTGCCCCAAAATG-3'
3	brain natriuretic peptide (BNP)	5'-GCAGAAGCTGCTGGAGCTGA-3'	5'-GATCCGGAAGGCGCTGTCT-3'
4	Sirtuin 1 (SIRT1)	5'-TGGCAAAGGAGCAGATTAGTAGG-3'	5'CTGCCACAAGAACTAGAGGATAAGA-3'

3. Results

3.1. C - reactive protein

On myocardial injury, CRP levels declared a significant increment (50 mg/L) as compared with healthy groups (4 mg/L). On the other hand, co-treatment by FLX, V.C. and combination of the two regimens showed a significant reduction of CRP values recording 5mg/L, 25mg/L, 35mg/L respectively. Data recorded indicates that co-treatment by FLX was improve CRP level to be near healthy group. Furthermore, data recorded that administration of V.C. improved CRP value than combination of two regimens (Fig. 1).

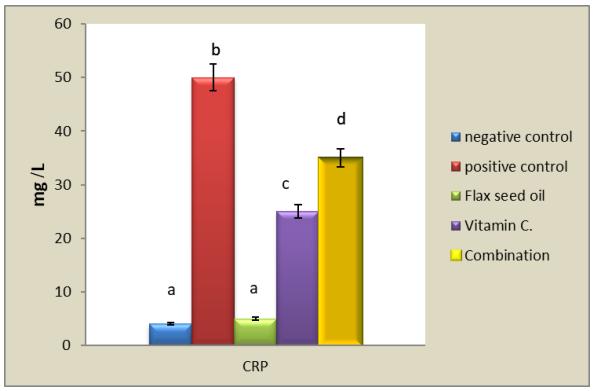

Fig.1: Co-treatment Effect of FLX oil, Vitamin C and combination of two regimens on serum C-creative protein (CRP) within arsenic trioxide toxicity. Metadata were expressed as means ± SEM (n=10). Groups with the same letters were non-significantly different from each other, meanwhile the others those have different letters were significantly different from each other. p <0.05 is deemed significant.

3.1 AST measurement

Upon arsenic toxicity in male rats, AST value demonstrated a significant increase (87.5unit/ml) as compared with negative control healthy groups (21.5unit/ml). On the other hand, co-treatment by FLX, V.C. and combination of the two regimens reported a significant reduction of AST level recording 50units/ml, 60unit/ml, 52.5 unit/ml respectively. Data recorded indicates that co-treatment by all treated regimens improve AST but not near to healthy group (Fig. 2).

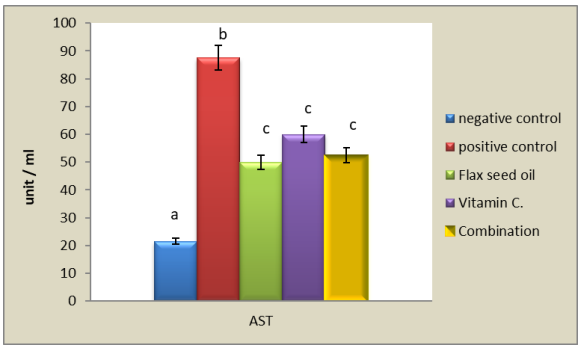

Fig.2: Co-treatment Effect of FLX oil, Vitamin C and combination of two regimens on serum AST within arsenic trioxide toxicity. Metadata were expressed as means ± SEM (n=10). Groups with the same letters were non-significantly different from each other, meanwhile the others those have different letters were significantly different from each other. p <0.05 is deemed significant.

3.2 Oxidative stress reduction

Arsenic toxicity on heart tissue caused an obvious oxidative stress appeared as a significant elevation in MDA level recording 11.5unit/ml as compared to healthy control group (5.88 unit/ ml). However, co-treatment with FLX, V.C. and combination of the two regimens reported a significant modulation of MDA value recording 6.5 unit/ ml, 7.05 unit/ ml and 5.06 unit/ ml respectively. The results indicated that combination of FLX and V.C. was the most regimens in modulating MDA values (Fig. 3).

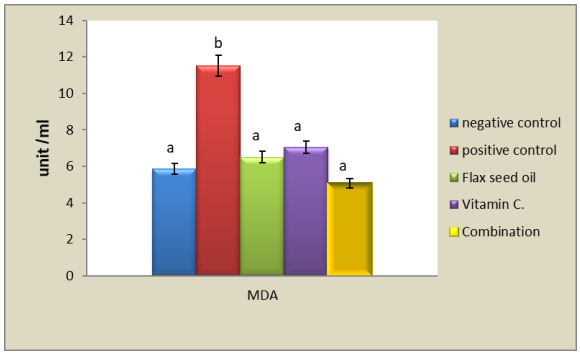

Fig.3: Co-treatment Effect of FLX oil, Vitamin C and combination of two regimens on serum MDA within arsenic trioxide toxicity. Metadata were expressed as means ± SEM (n=10). Groups with the same letters were non-significantly different from each other, meanwhile the others those have different letters were significantly different from each other. *p* <0.05 is deemed significant.

3.3 Modulation of Cardiac myosin heavy chains

Arsenic trioxide intoxicated group declared a significant reduction in the gene expression of α-MHC (0.47 fold change) as compared to negative control group. Furthermore, co-treatment by FLX oil with or without vitamin C significantly upregulated α-MHC gene expression (0.8 & 0.75 fold change respectively). Administration of vitamin C declared nonsignificant improvement in α-MHC gene expression (0.5 fold change) as compared healthy group.

On the other hand, a significant elevation in β-MHC (4.18 fold change) was reported upon arsenic trioxide intoxication as compared to negative control group. Co-treatment by FLX oil declared a significant downregulation of β-MHC mRNA gene expression (3.7 & fold change).On the other hand, nonsignificant downregulation of β-MHC mRNA gene expression was observed upon vitamin C co-treatment or combined by FLX oil (3.95 & 3.8 fold change respectively) (Fig.4).

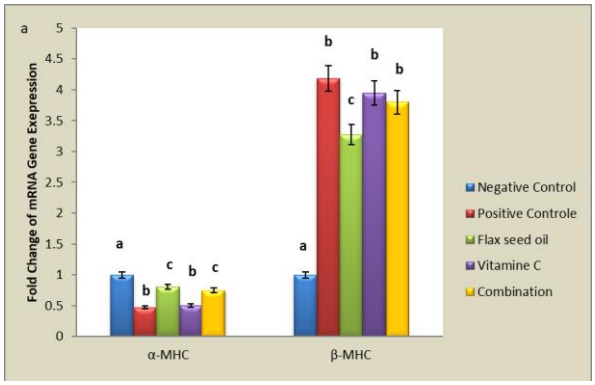

Fig. 4: Effect of arsenic trioxide intoxicated rats on mRNA gene expression of α-MHC and β-MHC. Metadata were expressed as means ± SEM (n=10). Groups with the same letters were non-significantly different from each other, meanwhile the others those have different letters were significantly different from each other. *p* <0.05 is deemed significant.

3.4 Modulation of Brain Natriuretic Peptide (BNP)

From Data recorded, a significant elevation in BNP mRNA gene expression was recolored upon arsenic trioxide intoxication (2.03 fold change) as comparing to negative control group. Furthermore, co-treatment of FLX oil, vitamin C or combination of two regimens recorded a significant downregulation in BNP mRNA gene expression (1.06, 1.15&1.32 respectively) with the superiority of FLX co-treatment group to modulate BNP mRNA gene expression (Fig 5).

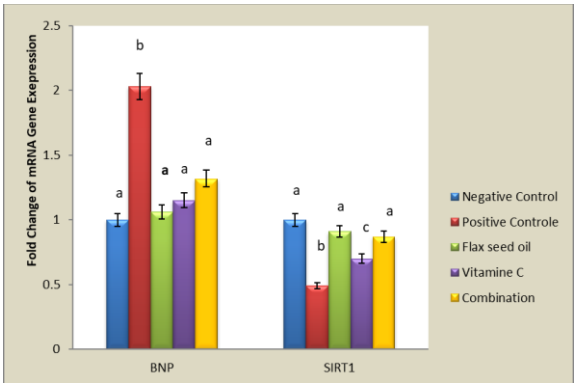

Fig. 5: Effect of arsenic trioxide intoxicated rats on mRNA gene expression of BNP and SIRIT. Metadata were expressed as means ± SEM (n=10). Groups with the same letters were non-significantly different from each other, meanwhile the others those have different letters were significantly different from each other. *p* <0.05 is deemed significant.

3.5 Modulation of Sirtuin1 (SIRT) mRNA gene expression

Gene expression of SIRT1 reduced significantly in arsenic trioxide intoxicated group recording 0.49 fold change as compared to negative control. However, this reduction recorded a significant upregulation in co-treated groups. Data recorded declared that FLX oil group was improved to be near healthy group upon FLX co-treatment alone or combined by vitamin C (0.91&.87fold change respectively). Meanwhile co-treatment by vitamin C only recorded low improvement percentage (0.7 fold change) (Fig 5).

4. Discussion

Arsenic trioxide has been established as an important compound for the management of acute leukemia in recent years but with association with some critical counteractive phenomena, especially cardiac functional abnormalities. Chemotherapeutic treatment of cancer was previously reported to induce

cardiac toxicity[45]. The main aim of the current study was to realize the cardiac safety of FLX oil and vitamin C against arsenic trioxide induced cardiac injury in rat model.

Results from the present work indicated that arsenic trioxide oral administration showed a significant increment in of oxidative stress markers and liver function enzyme AST. Cardiac Malondialdehyde (MDA) levels and CRP declared a significant increase upon arsenic treatment. The current investigation supported earlier findings that arsenic intoxication has a negative impact on antioxidant defense system and oxidative stress indicators [39, 41]. As recorded previously, arsenic trioxide increases lipid peroxidation and suppresses antioxidants [46, 47]. MDA levels are indicators of free radical formation and it produced as a breakdown product of the major chain reactions leading to the oxidation of polyunsaturated fatty acids [48]. The involvement of MDA was found in measuring oxidative stress and other forms of biological damages [38].

The current findings are consistent with the previously observed effects of arsenic trioxide toxicity, which include release of cytochrome C and apoptosis-inducing factor (AIF), reduction of cellular endogenous antioxidant reserve, and eventual apoptosis.[49, 50]. Co-treatment with FLX oil significantly reduced oxidative stress (measured as serum MDA).

In the current research, Arsenic administration was associated with increment CRP level. This result is consistent with another study and confirms earlier evidence linking arsenic exposure to cardiovascular disorders. [39].Mainly CRP measurement was reported as a strong cardiovascular diseases predictor and is probably not only a biomarker of inflammation, but an important agent in pathogen of cardiovascular disease [51].

It is well known that FLX oil contains different essential compounds such as the omega-3 andn-3 polyunsaturated fatty acid (PUFA) that reduce oxidative stress which induced in experimental animals[33]. As previously demonstrated, supplementation of n-3 PUFA may have a protective effect against myocardial infarction in experimental animals [52].

Different previous studies investigated the potential role of FLX as anticancer, anti-inflammatory, and anti-atherogenic agent [33, 52].The current research declared an obvious significant improvement in CRP, AST and oxidative stress measured biomarkers upon FLX oil administration to be near normal values. The detected result was in the agreement with the previous investigation that declared an administration of FLX oil decreased arsenic sedimentation in heart rats in addition to regeneration of damaged cardiac tissue as compared to those treated by arsenic only[7].

The genetic association responsible for protection against arsenic trioxide cardiovascular toxicity has been investigated previously. This association may play an essential role in cardiovascular pathogenesis. Herein, all the experimental molecular as well as biochemical investigations proved toxic effects produced by arsenic trioxide. A remarkable reduction was observed in α-MHC gene expression upon arsenic trioxide intoxication on the other hand a significant increment in β-MHC level in arsenic trioxide intoxicated group. These results highlight that arsenic trioxide induces pathological hypertrophy in the heart. Different previous studies reported that cardiac myosin heavy chain isoforms turn from α to β form in the hypertrophied, stressed, and failed heart where; myocardial mRNA expression of α-MHC and β-MHC were reduced and increased respectively, within arsenic trioxide exposure [53-55]. On the other hand, FLX co-treatment recorded a significant modulation in the expression of both α-MHC and β-MHC. The obtained results proved the beneficial impact of FLX seed oil in the protection against cardiovascular disease due to the presence of omega-3 [40, 56].

BNP was previously recorded as an essential biomarker responsible for heart dysfunction where, it released in the ventricle based on ventricular hemodynamic alternations which can reflect ventricular dysfunction [57]. In the present study, gene expression of BNP levels was significantly elevated up on arsenic trioxide toxicity on the other hand, an obvious improvement was declared upon treatment by FLX as well as vitamin C or combination of the two regimens. Different earlier researches revealed a significant alteration in BNP gene expression between cardiac intoxicated group and healthy one [18-20].

Autophagy has been shown to contribute to cell death suggesting its protective role. Thus, the functional significance of autophagy is often associated with apoptosis which is the main form of programmed cell death. Autophagy and apoptosis interaction can act as partners to induce cell death, autophagy can block apoptotic cell death by activation of cell survival, or autophagy can permits apoptosis in the cell without leading to death in it [22].

In the current study, significant association of cardiovascular toxicity in SIRT1 gene expression was recorded up on arsenic trioxide administration. An obvious reduction was reported of SIRT1 level upon arsenic trioxide intoxication. However, a detectable upregulation was revealed via co-treatment of FLX, vitamin C and combination of the two regimens. SIRT1 was previously reported to play a critical role in different physio pathological processes, stress, metabolism and aging in addition to its protection role against cardiovascular diseases via modulation of both pro-inflammatory and pro-apoptotic signaling pathways [25].

Data from the current study illustrated that co-treatment of V.C. with arsenic reduced an oxidative stress induced by arsenic toxicity. V.C. is a water-soluble antioxidant molecule that plays an essential role in the antioxidant defense system. Through its ability to donate electrons, it can reduce free radicals and in hence improve health of the body. The potential role of V.C. in the protection heart tissue agonist myocardial injuries was previously detected [58, 59]. Furthermore different studies reported that combination of treatment with V.C. improve different physiological status of the body and reduced oxidative stress [60-62].

5. Conclusions

In conclusion, FLX could be a promising therapeutic regimen against myocardial injury associated by arsenic toxicity. Furthermore, the beneficial role could be enhanced by combination of FLX with V.C. Cardio-protective impact of FLX may be due to the presence of sufficient amount of omega-3 fatty acid to maintain the official balance between pro-oxidant and antioxidant defense system. Based on our biochemical and molecular findings, it could be recommended that, dietary supplementation of FLX in arsenic trioxide treatment may modulate its cardio toxic effect.

Ethics approval and consent to participate
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Availability of data and material
No additional data or information is available for this publication.

Competing interests
The authors declare that they haven't any competing interest.

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Author contribution statement
Rehab M Abdel Megeed: Planed and designed the experiments; shared in performing the experiment; Analyzed (biochemical parameters and RT-PCR gene expression) and interpreted the data; contributed re-agents, materials, analysis tools or data; wrote the paper.
Mai O Kadry: shared in performing the experiment, contributed re-agents, analyzed (biochemical parameters and RT-PCR gene expression) and interpreted the data.

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