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Targeting Oxidative Stress: A Comparative Study on the Effects of Doxorubicin on Antioxidant Enzymes Activities in Heart and Tumor Tissues in Mice

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Abstract: ***Background:*** *Oxidative stress is a key component in linking environmental toxicity to the multistage carcinogenic process. Reactive oxygen species (ROS) are generated in response to both endogenous and exogenous stimuli. To counterbalance ROS-mediated injury, an endogenous antioxidants defense system exists; however, when oxidation exceeds the control mechanisms, oxidative stress arises. Doxorubicin (DOX) belongs to the class of anthracycline antibiotics that is widely used in the treatment protocols of a wide range of malignancies. The major deleterious effect of doxorubicin use is the possible occurrence of cardiotoxicity.* ***Purpose:*** *The present study aimed to investigate the effect of DOX on antioxidant enzymes activity in the cardiac and tumor tissues in mice.* ***Methods:*** *Sixty BALB/c male mice were used in this study. Except for mice in the control group, each mouse was implanted subcutaneously with 0.2 ml of the ascites fluid containing 1x106 Ehrlich carcinoma cells (ECCs) into the thigh of the hind limb. Mice were divided into three groups (20 mice per group) as follow: Control group, in which mice received an intraperitoneal (i.p.) injection of 0.2 ml normal saline once weekly on days 0, 7, 14, 21 (for 21 days), Solid Ehrlich carcinoma (SEC) control group, in which mice received an intraperitoneal injection of 0.2 ml normal saline once weekly on days 0, 7, 14, 21 (for 21 days) starting one hour after tumor inoculation, Doxorubicin (DOX+ SEC) group, in which mice received DOX (4 mg/kg, i.p.) once weekly on days 0, 7, 14, 21 (for 21 days) starting one hour after tumor inoculation. Serum creatine kinase (CK-MB), lactate dehydrogenase (LDH) and troponin I (cTn-I) levels activities, which are cardiac function markers were determined. Also, the levels of malondialdehyde (MDA) was assessed and enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) were assessed to determine the effect of DOX on antioxidant enzymes activity in the cardiac and tumor tissues in mice.* ***Results:*** *Administration of DOX to ECCs-bearing mice resulted in a significant increase in serum levels of CK-MB, LDH, cTnI, tissue SOD, CAT, GR and GPx with significant decrease in tissue MDA compared to SEC group. Administration of DOX to ECCs-bearing mice resulted in a significant decrease in cardiac tissue SOD, CAT, GR and GPx with significant increase in tissue MDA compared to control group.* ***Conclusion:*** *In tumor tissues, DOX was found to increase the activity of antioxidant enzymes, whereas in heart tissues, it reduced their activity. This variation might be due to the specific type of tissue affected by DOX. In cardiac tissues, DOX affects the cardiac adriamycin-responsive protein (CARP), which is exclusively found in these tissues and functions as a negative regulator of cardiac-specific gene expression.*

Keywords: Oxidative stress, Tumor, Reactive oxygen species (ROS), Antioxidant enzymes, Doxorubicin, Cardiotoxicity.

1. Introduction

Cardiovascular diseases and cancer represent the first and second cause of death in industrialized countries. These two conditions may become synergistic if we consider the cardiovascular complications of anticancer therapies [1].

Cancer is the most worrisome health problem that has received worldwide attention in the past decades. Currently, it is the second leading cause of death in developing countries after cardiovascular mortality [1]. More than 14 million new cancer cases occurred worldwide in 2012, according to the International Agency for Research on Cancer (IARC). The number of cancer deaths increased by 1.5 million, from 6.7 in 2002 to 8.2 million in 2012 [2]. By 2030, the global burden is expected to reach 21.7 million cancer cases and 13 million cancer deaths [3]. It is accounting for about 10 million deaths in 2020. Its incidence increases yearly, compromising individuals, families, and communities physical and emotional health. It is estimated that in 2040 there will be around 30 million cases, an increase of 57% compared to 2020 [4].

Reactive oxygen species (ROS) are highly reactive molecules that are principally derived fromthe oxygen that is consumed in various metabolic reactions occurring mainly in the mitochondria, peroxisomes, and the endoplasmic reticulum. ROS include the superoxide anion (O2 ·−) and hydroxyl radicals (OH·) as well as nonradical molecules such as hydrogen peroxide (H2O2). H2O2 is the more stable and diffusible form of ROS, it is selectively reactive towards cysteine residues on proteins, and, in the low nanomolar range, it can control cellular signaling [5] as presented in Figure 1 [6].

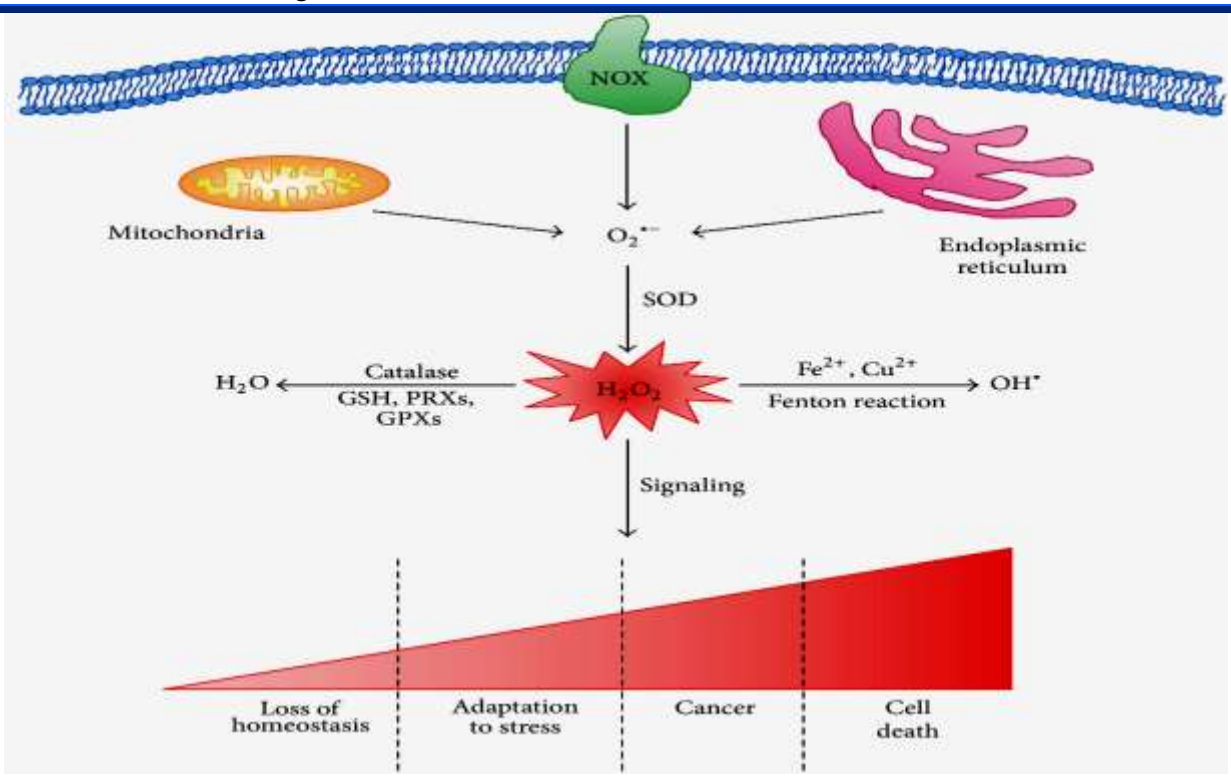

Figure 1: Redox homeostasis is a balance of ROS generation and elimination. Mitochondria, NAPH oxidase (NOX), and endoplasmic reticulumare the three major intracellular sources of ROS. Anion superoxide (O2·−) is the principal formof ROS and can be rapidly converted into hydrogen peroxide (H2O2) by superoxide dismutase (SOD). H2O2 can be catalyzed to hydroxyl radical (OH·) in the presence of Fe2+ or Cu2+ ions or be converted to H2O by catalase.The amount of H2O2 is decisive for the cell fate: low and intermediate levels of the peroxide stimulate loss of cell homeostasis and increased adaptation to stress leading to neoplastic transformation while high levels induce cell death [6].

ROS are mainly produced by the mitochondrial respiratory chain and also by enzyme-catalyzed reactions involving NADPH oxidase (NOX), xanthine oxidase, nitric oxide synthase (NOS), arachidonic acid, and metabolizing enzymes such as the cytochrome P450 enzymes, lipoxygenase, and cyclooxygenase [7]. ROS were first studied for tumorigenesis promoting activity in the mid-90s [8,9] however, their cellular homeostasis is essential for normal cell survival and proper cell signaling. Low ROS levels can activate signaling pathways in a regulatory manner that is essential for metabolic adaptation, differentiation and cellular proliferation [8]

Oxidative stress refers to the stress state caused by the unbalance between the weakening of body antioxidant defense system and the excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which is one of the fundamental causes for Dox-induced cardiotoxicity (DIC) [10]. Oxidative stress plays an essential role in cancer development and progression since high levels of reactive oxygen species (ROS) can trigger damage to biomolecules, promoting carcinogenesis [11]. On the other hand, this metabolic imbalance may result in cell death by different mechanisms, becoming the induction of oxidative stress a potential strategy in anticancer therapy [12, 13].

Doxorubicin (DOX) is one of the most widely prescribed antineoplastic drugs introduced over the past 50 years and remains the cornerstone for other targeted agents in standard tumor chemotherapy regimens [14-16]. It is used in the treatment of several types of human malignancies including hematological malignancies, solid tumors, soft-tissue sarcomas and breast carcinoma [17, 18]. The therapeutic potential of DOX is achieved through the processes of intercalating into DNA, inhibiting topoisomerase II, preventing DNA and RNA synthesis [19]. However, its clinical applications are relatively restricted due to its detrimental side effects that include cardiotoxicity [20].

Cardiac dysfunction triggered by doxorubicin (DOX) has long been known as the main form of anticancer drug induced cardiotoxicity, being characterized by massive accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as central mechanisms [21–23]. Oxidative stress Production of free radicals is the primary concern that produces cardiac muscle cell injuries after Dox administration [24]. The mechanism that mediates doxorubicin-induced cardiotoxicity is unclear; however, it might be related to oxidative stress, produced by increased levels of free radicals [25], intracellular iron [26, 27], and decreased

levels of antioxidants [28]. This oxidative stress causes increased intracellular calcium [28], and acceleration of lipid peroxidation [29, 30]. Moreover, increased ROS level suppresses the expression of nuclear factor erythroid 2-related factor (Nrf2) (Figure 2), which increases the cellular susceptibility to oxidative stress and apoptosis [31]. Increased oxidative stress leads to ROS generation, which causes damage to the heart muscle and arises due to the reduced level of antioxidants and sulphhydryl groups [32]. Dox-induced cardiotoxicity is usually accompanied by raised troponin, creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH) levels in the serum [33].

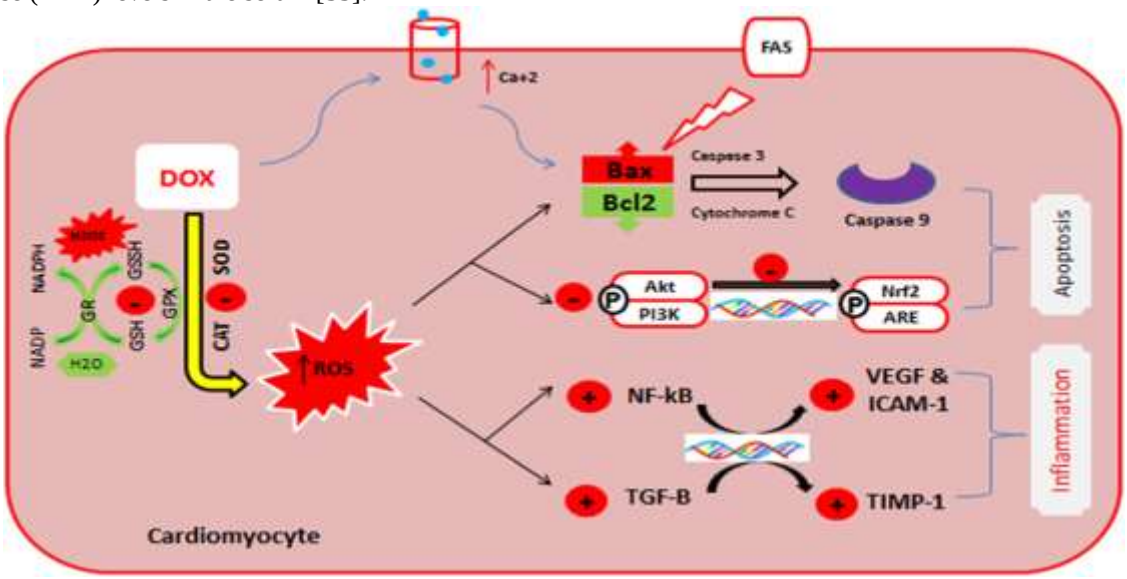

Figure. 2 shows the pathogenic effects of DOX on the molecular level and the central role of reactive oxygen species in DOX-induced cardiomyopathy [34].

High level of ROS and RNS may activate cytotoxic signalling leading to DNA damage, mitochondrial dysfunction, attenuation in protein synthesis, and deregulation of intracellular calcium homeostasis [35–38], which causes apoptosis. Apoptosis occurs in the cardiomyocytes and in the endothelial cells with the activation of caspase [39]. There is a strong correlation between cumulative doses of DOX and incidence and severity of DIC, in addition, age and previous cardiovascular disease were found to increase the incidence of DIC. Severe cardiomyopathy cause progressive heart failure and irreversible cardiac dysfunction, even death, severely affecting the quality of survival of cancer survivors [40].

ROS have a variety of negative effects in the cell, the multiple biochemical reactions in which oxygen is involved leads to the formation of reactive toxic intermediates that may cause DNA damage. Because oxidative damage to DNA can cause mutations; and mutations are known to cause cancer, much effort has been devoted to study the role in carcinogenesis of oxidative DNA damage [41, 42].The flowchart in Figure 3 briefly summarizes the more dangerous effects of ROS in oxidative stressed cells [43].

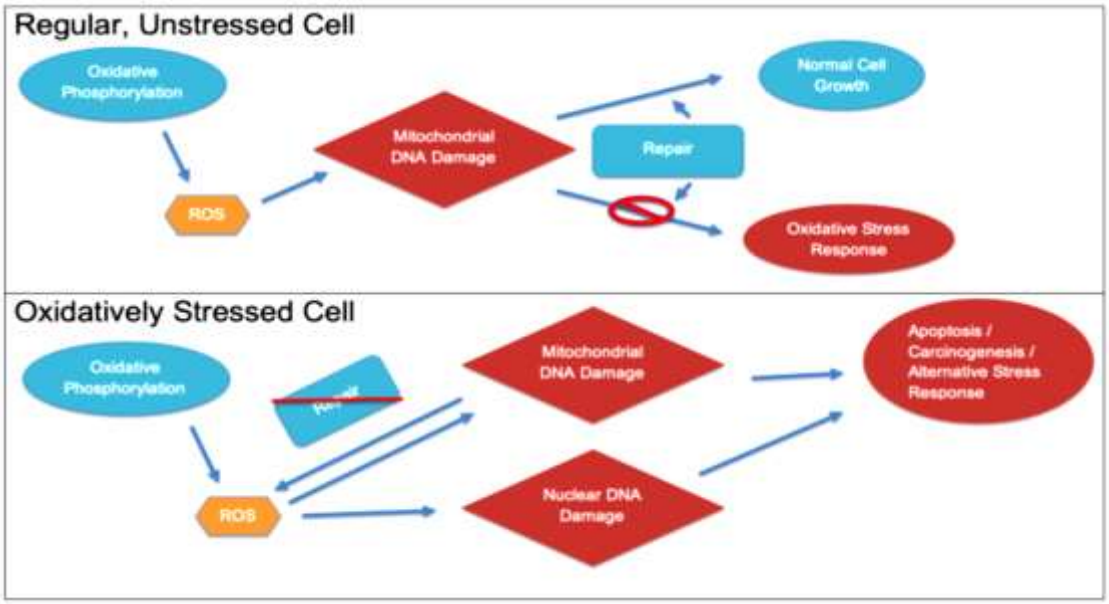

Figure 3: Different effects of ROS in a normal vs. oxidatively stressed cell. Note that in the normal cell, the effects of ROS are quickly repaired, but that is not so in a stressed cell [43].

The cell utilizes antioxidants to regulate ROS levels. Antioxidants are a diverse array of molecules, both enzymatic and nonenzymatic, that can be found in the extracellular matrix, mitochondria, and cytoplasm of the cell [44]. Oxidant/antioxidant balance has been suggested as an important factor for initiation and progression of cancer [42]. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ (endogenous) or externally supplied through foods and/ or supplements (exogenous). Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS; and therefore can enhance the immune defense and lower the risk of disease and cancer [45, 46].

Endogenous antioxidant compounds in cells can be classified as enzymatic antioxidants and non-enzymatic (metabolic and nutrient) antioxidants. The major enzymatic antioxidants directly involved in the neutralization of ROS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) [47, 48].

The role of ROS in cancer initiation and development offers 2 main avenues of cancer treatment possibilities. The first is through antioxidants, the natural enemy of ROS. However, several cancer treatments actually involve increasing ROS production [49]. Several antioxidants are used by the cell for the conversion of various ROS into non-toxic forms, and this is why they have been looked to as a potential solution for oxidative stress problem. The other potential solution utilizes the naturally high levels of ROS in cancerous cells to selectively trigger mass apoptosis in tumors [49]. In addition to, Up-regulated ROS levels and down-regulated cellular antioxidant enzymes lead to different malignancies through different molecular factors like nuclear factor kappa B (NF-κB) and nuclear factor (erythroid-derived 2)-like-2 factor (NRF2) [50].

Therefore, the present study was designed to compare the effect of doxorubicin on the activity of antioxidant enzymes in cardiac and tumor tissues by targeting oxidative stress in mice.

2. Materials and Methods

2.1. Drugs Used

Doxorubicin (DOX) was commercially available in powder form for injection purchased from Carlo Erba, Turkey. It was dissolved in normal saline and administered by intraperitoneal injection once weekly for 4 weeks [51].

2.2. Solid Ehrlich carcinoma (SEC) tumor model

1 x 10^6 of Ehrlich carcinoma cells (ECC) obtained from the pharmacology and experimental oncology unit of the national cancer institute, Cairo University, Egypt were implanted subcutaneously into the right thigh of the hind limb of mice. A palpable solid tumor mass was developed within 12 days [52].

2.3. Animals

BALB/c mice weighing about 20–25 grams, obtained from the animal house of the faculty of medicine, Tanta University, Egypt. Animals were kept in individual metabolic cages at 22 ºC, 55% relative humidity and 12/12 hours light-dark cycle through the whole period of the study. The protocol of this study was conducted following the Helsinki declaration of animal ethics [53].and was approved by the Research Ethics Committee of Faculty of Medicine, Tanta University,

2.4. Experimental Design

Sixty BALB/c male mice were used in this study. Except for mice in the control group, each mouse was implanted subcutaneously with 0.2 ml of the ascites fluid containing $1x10^6$ ECCs into the right thigh of the hind limb of mouse.

The day of implantation of ECCs was considered as the zero point (day 0) of the experiment. The total period of the experiment was 42 days. Mice were randomly divided into three equal groups (20 mice per each group) as follows:

Group 1: Control group, in which mice received intraperitoneal (i.p.) injection of 0.2 ml normal saline once weekly on days 0, 7, 14, 21 (for 21 days).

Group 2: Solid Ehrlich carcinoma (SEC) control group, in which mice received intraperitoneal injection of 0.2 ml normal saline once weekly on days 0, 7, 14, 21 (for 21 days) starting one hour after tumor inoculation [54].

Group 3: Doxorubicin (DOX) group, in which mice received DOX (4 mg / kg, i.p.) once weekly on days 0, 7, 14, 21 (for 21 days) starting one hour after tumor inoculation [55].

At the end of the study, 42 days after tumor inoculation and first injection of DOX. Then the blood samples were collected. Mice were sacrificed and their hearts and tumor tissues were excised for further investigation.

2.5. Assessment of Cardiac Function Tests in Blood Samples

Blood was withdrawn from the orbital sinus of mouse under light ether anesthesia. Serum was separated immediately by centrifugation at 4000 rpm for 10 minutes, which was utilized for assessment of lactate dehydrogenase (LDH) using kits supplied by STANBIO, USA according to Buhl and Jackson [56]. Kits purchased from STANBIO, USA, were utilized for quantification of the levels of serum creatine kinase (CK-MB) [57], and serum troponin I (cTn-I) using ELISA kits purchased from Sigma Aldrich Co. according to the instructions of the manufacturer [58].

2.6. Processing and Preparation of Tumor and Cardiac Tissues

Mice were euthanized; the tumor and heart of mice were immediately extracted out and freed from the adjacent tissues, washed with cold saline to remove any excess blood, blotted to dry on filter paper and then weighed. A portion of extracted tumor and heart tissues was homogenized by a Branson sonifier (250, VWR Scientific, Danbury, CT, USA) and the homogenate was centrifuged at 3000 rpm for 10 min. The resulting supernatant was utilized for exploration of the levels of the biochemical parameters in the specimens of tumor and cardiac tissues. The other portion of tumor and heart tissues was processed for further histopathological and immunohistochemical examinations.

2.7. Evaluation of Oxidative Stress Parameters Content in Tumor and Cardiac Tissues

The intracellular antioxidants in tumor and cardiac tissues were measured, such as catalase (CAT) according to Higgins et al., [59]. Superoxide dismutase (SOD) according to Marklund and Marklund [60]. glutathione reductase (GR) using kits supplied by Sigma Aldrich Co., USA, according to the instructions of the manufacturer, glutathione peroxidase (GPx) was determined using BIOXYTECH GPx-340TM Assay kit produced by OXIS International, Inc., USA according to Rotruck *et al.,* (1973) [61].

2.8. Lipid Peroxidation (LPO) Assay

The malondialdehyde (MDA) content was estimated to evaluate the peroxidation of lipids. Levels of MDA in tumor and cardiac tissue were measured using Uchiyama and Mihara method [62], according to the manufacturer's directions. This method depends on the fact that MDA reacts with TBA producing thiobarbituric acid reactive substance (allegedly a [TBA] 2 – Malondialdehyde adduct) a pink chromogen.

2.9. Statistical Analysis of the Obtained Data

For statistical analysis, the Statistical Package for the Social Sciences (SPSS) version 16.0 was used. Parameters were shown with mean ± Standard error of mean (SEM). Multiple comparisons were performed using one way analysis of variance (ANOVA) and nonparametric followed by Tukey-Kramer test for post hoc analysis, as appropriate. Unpaired t-test and Mann-Whitney test were used to compare between two different treatment groups. Differences between the means of the different groups were considered significant at a level of $p\text{-value} < 0.05$.

3. RESULTS

3.1. Effect of different treatments on serum LDH, CK-MB and troponin I.

DOX resulted in significant increase in serum LDH, CK-MB, and troponin I compared to the control and SEC groups (Figure. 4-6).

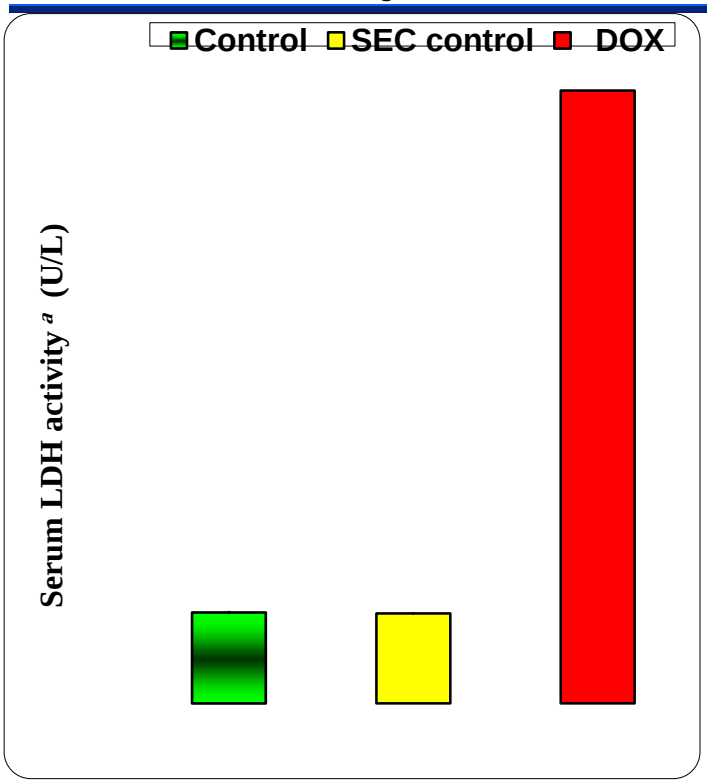

Figure. **4:** Effect of different treatments on serum LDH in the studied groups

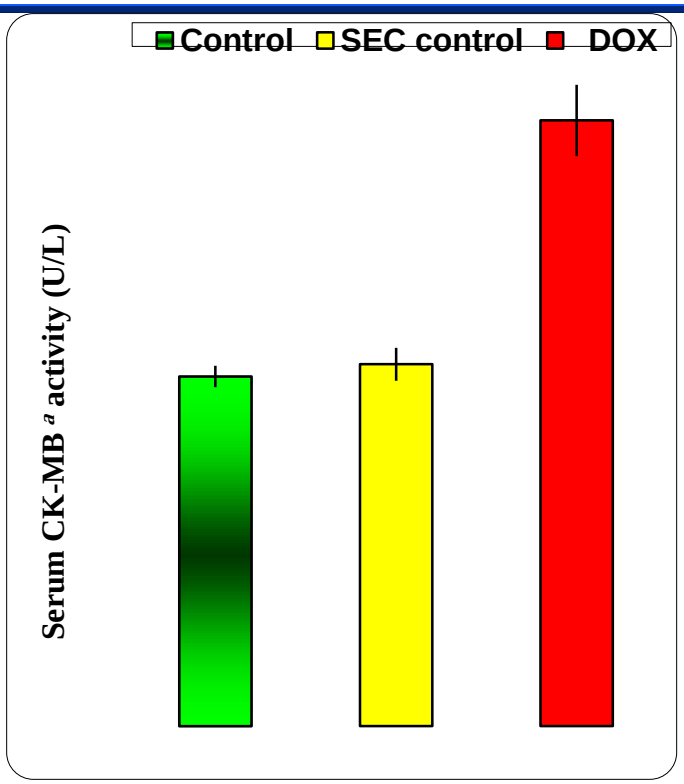

Figure. **5:** Effect of different treatments on serum CK-MB in the studied groups

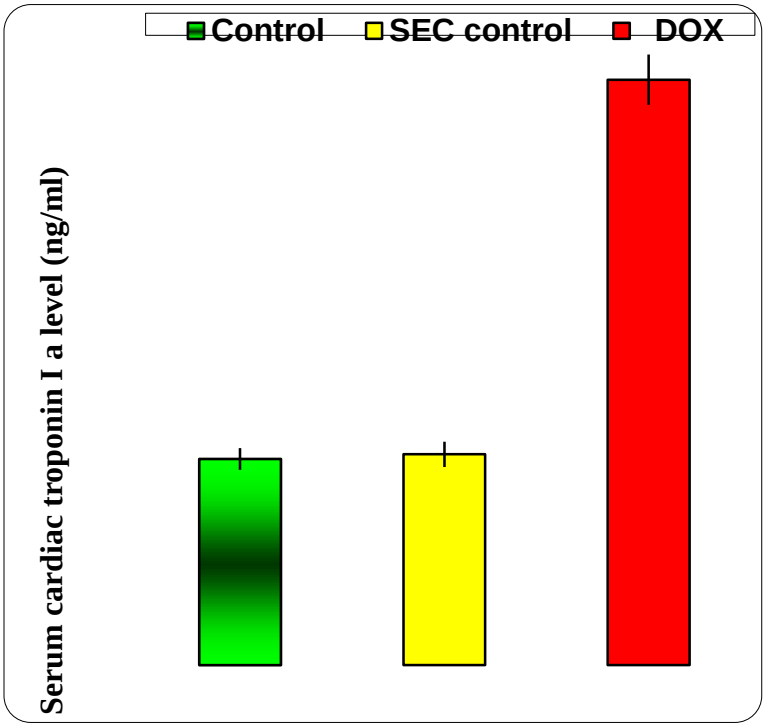

Figure. **6: Effect of different treatments on serum cardiac troponin I in the studied groups**

3.2. Effect of different treatments on tumor tissue antioxidant status

Subcutaneous implantation of ECC resulted in significant decrease in tumor tissue CAT, SOD, GR and GPx with significant increase in tissue MDA compared to the control group (Figure.7-8). DOX resulted in significant increase in tumor tissue CAT, SOD, GR and GPx with significant decrease in tumor tissue MDA compared to SEC **control** group (Figure.9-10).

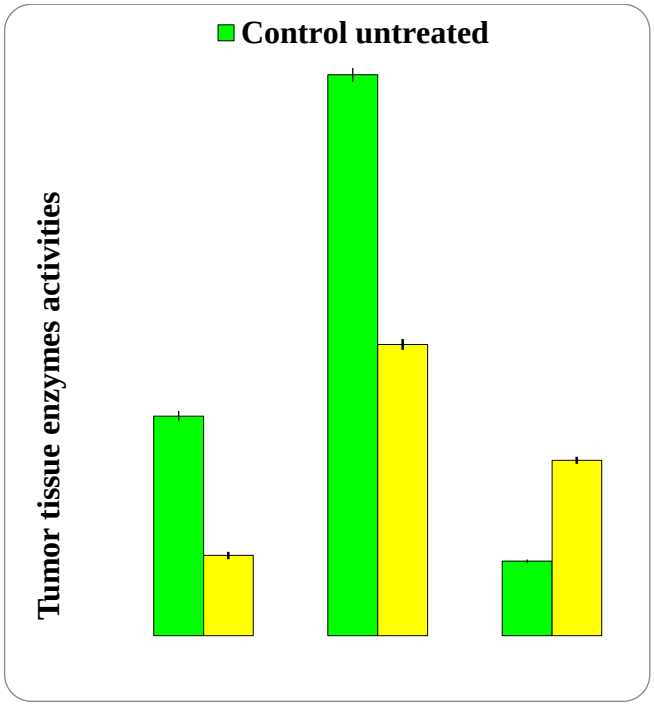

Figure. 7: Effect of Subcutaneous implantation of ECC on tumor tissue SOD, GR, and MDA in the studied groups

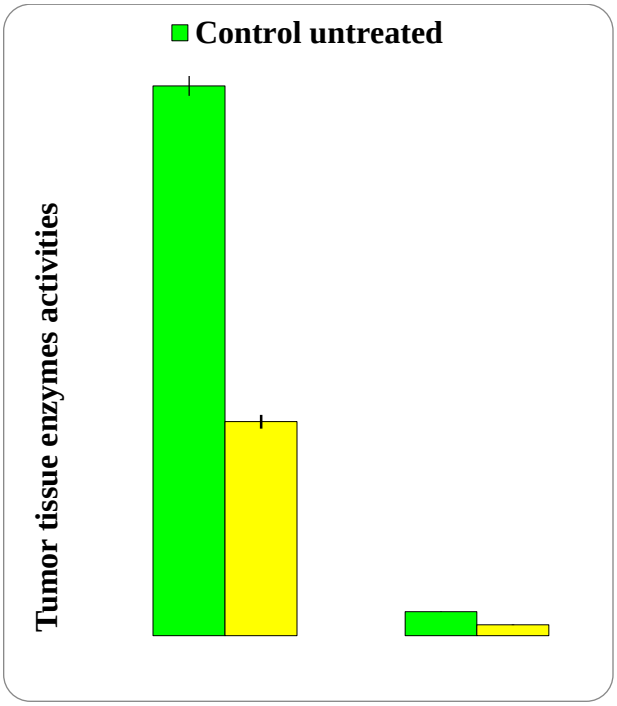

Figure. 8: Effect of Subcutaneous implantation of ECC on tumor tissue CAT and GPx in the studied groups

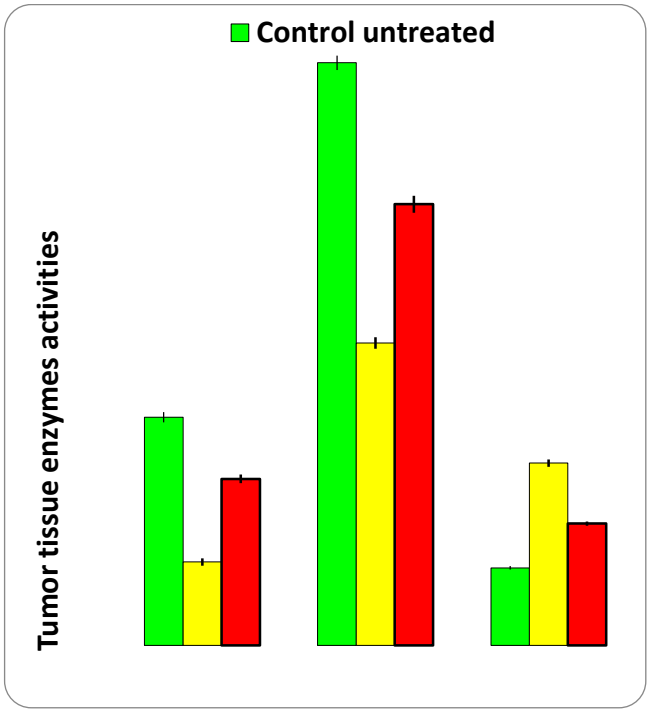

Figure.9: Effect of different treatments on tumor tissue SOD, GR, and MDA in the studied groups

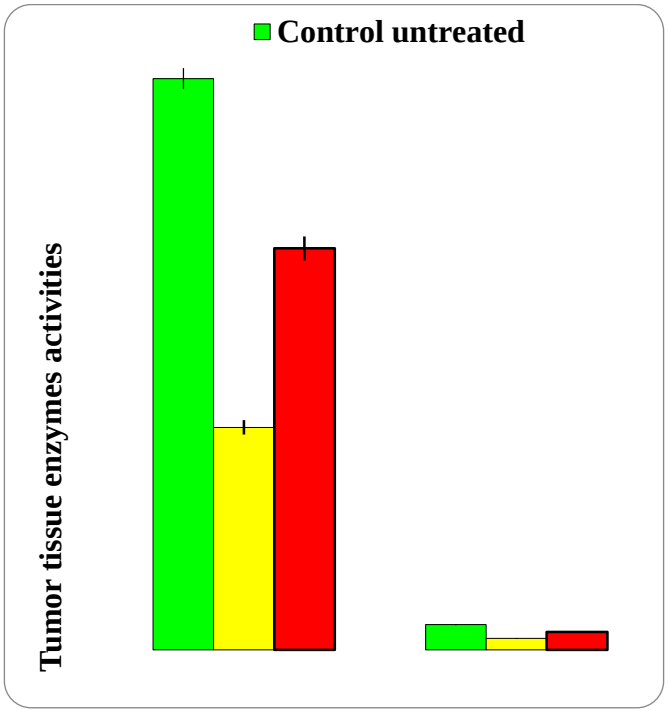

Figure.10: Effect of different treatments on tumor tissue CAT and GPx in the studied groups

3.3. Effect of different treatments on the cardiac antioxidant status

DOX resulted in significant decrease in CAT, SOD, GR and GPx levels in cardiac tissues with significant increase in cardiac tissue MDA compared to the control and SEC groups (Figure. 11-12).

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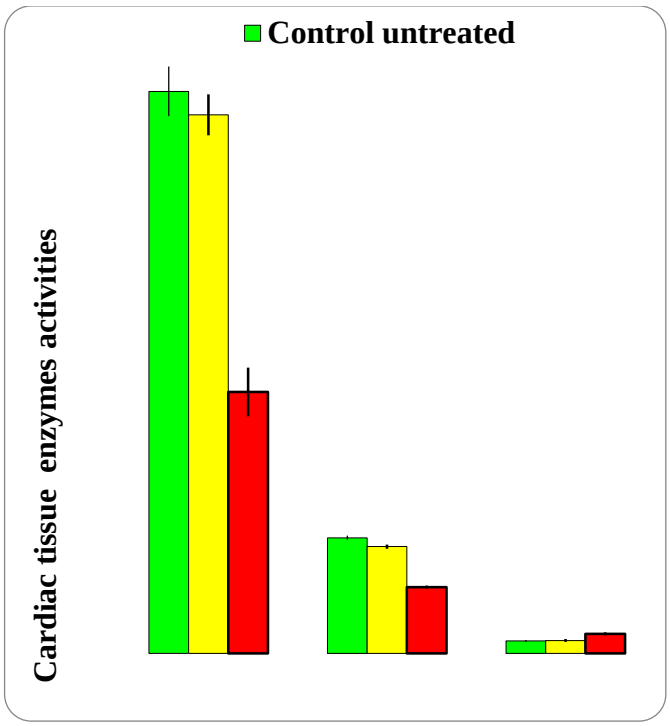

Figure. 11: Effect of different treatments on cardiac tissue SOD, GR, and MDA in the studied groups

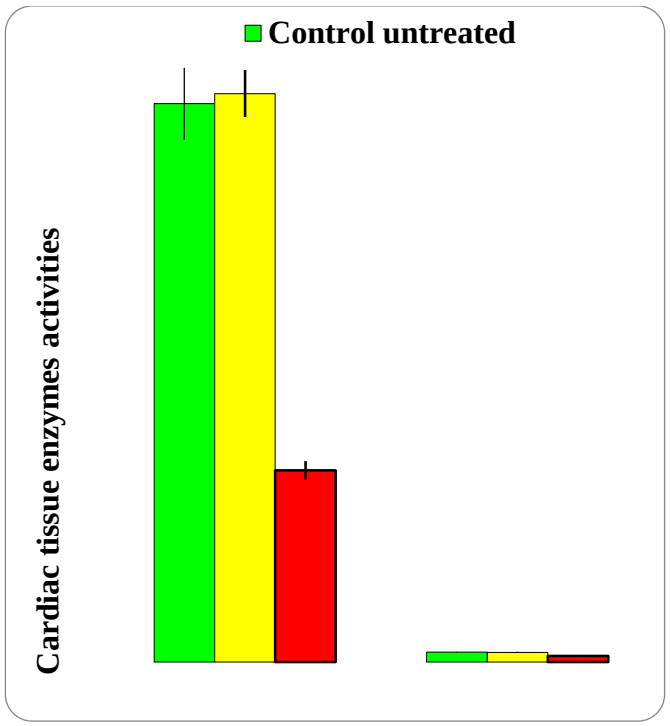

Figure. 12: Effect of different treatments on cardiac tissue CAT and GPx in the studied groups

4. Discussion

DOX is an effective chemotherapeutic agent but is known to cause cardiotoxicity, which can lead to heart failure. The cardiotoxic effects are primarily due to increased production of reactive oxygen species (ROS) and oxidative stress in heart tissues. [63].

In the present study, implantation of ECCs resulted in significant decrease in tumor tissue CAT, SOD, GR and GPx with significant increase in tissue MDA compared to the control group. These results were in agreement with Zahran et al., [64].and Kabel et al., [65]. It is documented that oxidative stress was involved in cellular processes ranging from apoptosis to cell proliferation and carcinogenesis. Moreover, induction of cell proliferation, decreased apoptosis and oxidative DNA damage had been proposed to be predisposing factors to carcinogenesis [66]. Pizzimenti et al., [67] reported that reactive oxygen species and lipid peroxidation products can affect the growth of cancer cells, possibly through regulation of cancer cell proliferation and the expression of the oncogenes. Poljsak et al., [68] reported that up-regulation of the antioxidant enzymes such as SOD in cancer cells resulted in mitochondrial accumulation of H_2O_2, which in turn damages DNA, induces apoptosis and inhibits tumor growth.

In the present study, DOX resulted in significant improvement in tumor tissue CAT, SOD, GR and GPx with significant decrease in MDA compared to the SEC group. These results were in accordance with El-Dayem et al. [69] and Osman et al. [70]. DOX was thought to interact with DNA leading to disruption of topoisomerase-II-mediated DNA repair [71]. It was reported that DOX enhances apoptosis through increasing the activity of caspases. Moreover, Al-Harthi et al. [72] suggested that DOX inhibits Ras signaling that regulates cell growth and differentiation. Kang et al. [73] reported that DOX suppressed had a protective effect against tissue damage induced by oxidative stress which was in the same line with the results of our study. However, Thorn et al. [74] suggested that the effect of DOX on cancer cells is due to generation of free radicals which leads to lipid peroxidation, DNA damage, oxidative stress and induction of apoptosis.

In the present study, administration of DOX resulted in cardiotoxicity manifested by significant increase in serum LDH, CK-MB and troponin I with significant decrease in cardiac tissue CAT, SOD, GR and GPx with significant increase in cardiac MDA compared to the control and SEC groups. These changes were attributed to the damaging effects of reactive oxygen species generated by the interaction of DOX with iron together with inhibition of DNA topoisomerase II and stimulation of certain immune and inflammatory responses in the cardiac tissues [75, 76].

More recent studies have suggested that doxorubicin affects the expression of certain genes related to the generation of ROS with the end result of distortion of the normal architecture and functions of cardiomyocytes [77]. This was in accordance with the data obtained from the current study where mice injected with doxorubicin exhibited significant deterioration in cardiac functions, represented by the significant elevation in serum LDH, CK-MB, and troponin I, when compared to the control group. In addition, Koul *et al.,* [78] and Osman *et al.,* [79] have shown that, the elevation of the level of the different enzymes by DOX probably reflects that the drug induces cardiac toxicity, where LDH, CK-MB and troponin-I are rather specific for myocardial damage.

In the present study, there was controversy about the effect of DOX on oxidative stress in tumor and heart tissues. Although DOX increases the activity of antioxidant enzymes in tumor tissues, on the other hand, it was found to increase ROS production and induce oxidative stress in heart tissues. This might be attributed to the type of the tissue on which DOX acts. In the cardiac tissues, DOX was found to affect cardiac adriamycin-responsive protein (CARP).

CARP is a crucial protein in heart tissues, playing a significant role in maintaining cardiac function and responding to stress [80]. In addition, Zhang, et al., [81] indicated that CARP is essential for maintaining the structural integrity of the heart muscle, playing a key role in the proper organization of sarcomeres, which are the basic units responsible for muscle contraction. Stecyk et al., [82] and Vornanen et al., [83] have shown that, CARP acts as a transcription co-factor, regulating the expression of genes related to cardiac function and stress response. Additionally, CARP responds to oxidative stress—an imbalance between ROS production and the body's ability to detoxify these reactive species—thereby helping to protect cardiac tissues from damage. [84]. Treatment with DOX reduces CARP expression, weakening the heart's capacity to handle oxidative stress and leading to cardiotoxicity [80]. CARP plays a key role in modulating the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). These enzymes neutralize reactive oxygen species (ROS) and safeguard cardiac cells from oxidative damage. [85]. Reducing CARP expression due to DOX can elevate oxidative stress and lead to cardiotoxicity [86].

This may give an explanation to the results of the present study where administration of DOX resulted in (1) tumor tissues, DOX increases the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), glutathione peroxidase (GPx), and glutathione reductase (GRx). This is because cancer cells often have enhanced antioxidant defenses to protect themselves from oxidative stress. The increased activity of these enzymes helps neutralize reactive oxygen species (ROS) generated by DOX, allowing the drug to target cancer cells more effectively. (2) heart tissues, DOX induces oxidative stress in heart tissues by increasing the production of reactive oxygen species (ROS), which can damage cardiac cells. This oxidative stress can lead to cardiotoxicity, impairing heart function. CARP helps regulate the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), which neutralize ROS and protect cardiac cells. When DOX downregulates CARP expression, the heart's ability to manage oxidative stress is compromised, leading to increased oxidative damage and cardiotoxicity.

5. Conclusion:.

In tumor tissues, DOX was found to increase the activity of antioxidant enzymes, whereas in heart tissues, it reduced their activity. This variation might be due to the specific type of tissue affected by DOX. In cardiac tissues, DOX affects the cardiac adriamycin-responsive protein (CARP), which is exclusively found in these tissues and functions as a negative regulator of cardiac-specific gene expression.

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