



Therapeutic Effects of Liraglutide, Oxytocin and Granulocyte Colony-Stimulating Factor in Doxorubicin-Induced Cardiomyopathy Model: An Experimental Animal Study

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Abstract

Doxorubicin-induced (DXR) cardiomyopathy is a serious health issue in oncology patients. Effective treatment of this clinical situation still remains to be discovered. In this experimental animal study, we aimed to define therapeutic effects of liraglutide, oxytocin and granulocyte colony-stimulating factor in DXR-induced cardiomyopathy model. 40 male Sprague–Dawley rats were included to study. 32 rats were given doxorubicin (DXR) for cardiomyopathy model. DXR was administered intraperitoneally (i.p.) at every other day of 2.5 mg/kg/day at six times. Eight rats were taken as normal group and no treatment was performed. 32 rats given doxorubicin were divided into 4 groups. Group 1 rats were assigned to a placebo group and was given with a 0.9% NaCl saline solution at a dose of 1 ml/kg/day i.p. (DXR + saline), Group 2 rats were given with 1.8 mg/kg/day of Liraglutide i.p. (DXR + LIR), Group 3 rats were given with 160 µg/kg/day oxytocin i.p. (DXR + OX), Group 4 rats were given with 100 µg/kg/day filgrastim i.p. (DXR + G-CSF). All medications were given for 15 days. On day 16, under anesthesia, ECG was recorded from derivation I. After that, blood samples were taken by tail vein puncture for biochemical analysis. Finally, the animals were euthanized and the heart removed and prepared for immunohistochemical examination. All three treatments were shown to ameliorate the toxic effect of doxorubicin in cardiac tissue with the best results in DXR + OX group. DXR + OX group had the most preserved tissue integrity examined by light microscopy, least immune expression level of CASPASE-3 (5.3 ± 0.9) ($p < 0.001$) the highest ECG QRS wave voltage amplitude (0.21 ± 0.008 mV) ($p < 0.00001$) least plasma MDA (115.3 ± 19.8 nm) ($p < 0.001$), TNF-alpha (26.6 ± 3.05 pg/ml) ($p < 0.001$), pentraxin-3 (2.7 ± 0.9 ng/ml) ($p < 0.001$), Troponin T (1.4 ± 0.08 pg/ml) ($p < 0.001$), pro-BNP (11.1 ± 3.6 pg/ml) ($p < 0.001$) levels among all three treatment groups. Consistent with previous literature, we found that OX treatment decreased oxidative, apoptotic and inflammatory activity in DXR-induced cardiomyopathy rat model as well as provided better tissue integrity and better results in clinically relevant measures of ECG assessment, plasma Troponin T and pro-BNP levels. LIR and G-CSF treatment caused similar results with less powerful effects. Our findings suggest that with the best results in OX treatment group, all three agents including LIR and G-CSF attenuates DXR-induced cardiomyopathy in this rat model.

Keywords Doxorubicin · Cardiotoxicity · Liraglutide · Oxytocin · G-CSF · Inflammation

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Introduction

Doxorubicin is a well-known chemotherapeutic agent belongs to anthracycline group and is extensively used to treat numerous malignancies such as adenocarcinomas and lymphomas. Survival rates of these malignancies had improved since doxorubicin was introduced to chemotherapy protocols in 1960s [1]. However, it was discovered that doxorubicin caused acute cardiotoxicity characterized with prolonged QT interval in patients especially older than 65 years old [2]. Later, increased prevalence of heart failure was detected 10–15 years after chronic exposure to

doxorubicin and the severity of heart failure was correlated with the duration and dosage of doxorubicin exposed [3].

Liraglutide is a dipeptidyl peptidase -4 resistant glucagon like peptid-1 agonist that is used to treat type 2 diabetes mellitus and acts via enteral glucose intake-dependent incretin effect to lower blood glucose level. Aside from its glucose lowering effect, liraglutide was shown to exert neuroprotective and anti-oxidative features described by current literature. Liraglutide blocks lipid peroxidation, reverses AMP-activated protein kinase (AMPK) inhibition, lowers proinflammatory cytokine levels and causes less inflammatory state in recent animal studies conducted with different oxidating agents [4, 5].

Oxytocin is a peptide which is produced in supraoptical and paraventricular nuclei of hypothalamus and causes milk ejection during lactation and uterine contractions during labor. On the other hand, recent studies suggest that oxytocin has immunomodulatory, anti-inflammatory, anti-oxidative, anti-apoptotic features as well as it is shown to inhibit inflammatory cytokines and tissue damage in animal models of sepsis and neuropathy [6, 7].

Granulocyte colony-stimulating factor (G-CSF) is extensively used in hematologic malignancies to support myeloid proliferation after chemotherapy or bone marrow transplantation. In addition to its proliferative and differentiative features, recent animal studies suggest that it may have some beneficial effects in neurological disease. Although the mechanism of this situation is not clearly enlightened, researchers comment that anti-apoptotic, anti-inflammatory features and stem cell proliferative effect of G-CSF may have played important role [8, 9].

Doxorubicin causes myocardial damage by blocking anti-oxidative cellular mechanisms, causing accumulation of reactive oxygen species and increasing apoptosis of myocardocytes [10]. According to the current literature we hypothesized that liraglutide, oxytocin and G-CSF can ameliorate or prevent doxorubicin-induced cardiomyopathy by blockade of inflammatory pathways, inducing anti-oxidative enzymes and supporting the proliferation of stem cells in heart tissue.

Materials and Methods

Animals

In this study, 40 male Sprague–Dawley albino mature rats, weighing 200–220 g, were used. Animals were fed ad libitum and housed in pairs in steel cages having a temperature-controlled environment (22 ± 2 °C) with 12-h light/dark cycles. The experimental procedures employed in present study were approved by Animal Ethics Committee. All experiments were carried out according to the Guide for

the Care and Use of Laboratory Animals, as confirmed by National Institutes of Health (U.S.)

Experimental Protocol

40 male Sprague–Dawley rats were included to study. 32 rats were given doxorubicin (DXR) for cardiomyopathy model. DXR were administered intraperitoneally (i.p.) at every other day of 2.5 mg/kg/day at six times. The total doxorubicin dose was 15 mg/kg. Eight rats were taken as normal group and no treatment was performed. When these rats were given doxorubicin, three different medications were administered to prevent cardiomyopathy. For this, 32 rats given doxorubicin were divided into 4 groups. Group 1 rats were assigned to a placebo group and was given with a 0.9% NaCl saline solution at a dose of 1 ml/kg/day i.p. (DXR + saline), Group 2 rats were given with 1.8 mg/kg/day of Liraglutide i.p. (DXR + LIR), Group 3 rats were given with 160 µg/kg/day oxytocin i.p. (DXR + OX), Group 4 rats were given with 100 µg/kg/day filgrastim i.p. (DXR + G-CSF). All medications was given for 15 days. On day 16, under anesthesia, ECG was recorded from derivation I. After that, blood samples were taken by tail vein puncture for biochemical analysis. Finally, the animals were euthanized and the heart removed.

Assessment of ECG

ECGs were recorded on male Sprague–Dawley rats under anesthesia in the prone position. Electrodes consisted of 26-gauge needles placed subcutaneously for 1 cm. Standard limb leads were constructed from electrodes placed at the paws. Rats were anesthetized by combination of ketamine hydrochloride at a dose of 80 and 8 mg/kg of xylazine hydrochloric which were administered i.p. under anesthesia ketamine and xylazine are administered ip., ECG is taken in derivation I (D I) (Biopac MP 150). Data were evaluated using Biopac Student Lab Pro version 3.6.7 software, QRS wave amplitude was calculated peak to peak (mV). During the ECG recordings, rectal temperatures of the rats were monitored by a rectal probe and the temperature of each rat was kept at approximately 36–37 °C by heating pad.

Histopathological Examination of Heart Tissue

For histological and immunohistochemical studies, all animals were anesthetized by an i.p. of ketamin/xylazine and perfused with 200 ml of 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS). Formalin-fixed heart sections (5 µm) were stained with hematoxylin and eosin (H&E). All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope. Morphological analysis was assessed by computerized image

analysis system. The degree of damage in cardiac tissue was examined by light microscopy.

CASPASE-3 Immunoexpression

For immunohistochemical examination, sections were incubated with H_2O_2 (10%) for 30 min to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum for 1 h at room temperature. Subsequently, sections were incubated with primary antibodies for 24 h at 4 °C. Antibody detection was performed with the Histostain-Plus Bulk kit (Invitrogen) against rabbit IgG, and 3,3' diaminobenzidine (DAB) was used to visualise the final product. The photos were taken by Olympus C-5050 digital camera mounted on Olympus BX51 microscope. Brown cytoplasmic staining in cardiomyocytes was scored positive for *CASPASE-3*. The number of *CASPASE-3* (+) cells were assessed by systematically scoring at least cardiomyocytes 100 cells per field in 10 fields of tissue sections at $\times 10$ magnification objective.

Measurement of Plasma TNF- α

Plasma TNF- α levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit. The plasma samples were diluted 1:2 and TNF- α was determined in duplicate according to the manufacturer's guide.

Measurement of Plasma pro-BNP, Troponin T levels

Plasma *pro-BNP*, *Troponin T* levels were measured by using commercially available (ELISA) kit.

Determination of Lipid Peroxidation (MDA)

Lipid peroxidation was determined in plasma samples by measuring malondialdehyde (MDA) levels as thiobarbituric acid reactive substances (TBARS) [11]. Briefly, trichloroacetic acid and TBARS reagent were added to the tissue samples, then mixed and incubated at 100 °C for 60 min. After cooling on ice, the samples were centrifuged at 3000 rpm for 20 min and the absorbance of the supernatant was read at 535 nm.

Evaluation of Plasma Pentraxin-3 Levels

Plasma pentraxin-3 (PTX3) levels were measured in each 100 μ l sample by standard ELISA apparatus at 450 nm using a PTX3 kit. PTX3 levels were determined in duplicate according to the manufacturer's guide.

Statistical Analysis

Data analyses were performed using SPSS version 15.0 for Windows. The groups of parametric variables were compared by Student's *t* test and analysis of variance (ANOVA). The groups of nonparametric variables were compared by Mann–Whitney U test. Results were given as mean \pm standard error of mean (SEM). A value of $p < 0.05$ was accepted as statistically significant. $p < 0.001$ was accepted as statistically highly significant.

Results

According to findings above, DXR + Saline group has the worst tissue integrity with the highest level of *CASPASE-3* activity (14.6 ± 2.8) ($p < 0.000001$) which is an indicator of apoptosis. As indicators of tissue damage, increased oxidative and inflammatory state; ECG QRS voltage amplitude was found to be lowest (0.10 ± 0.004 mV) ($p < 0.000001$) plasma MDA (328.1 ± 43.2 nm) ($p < 0.001$), TNF-alpha (46.9 ± 4.1 pg/ml) ($p < 0.001$), pentraxin-3 (4.7 ± 1.1 ng/ml) ($p < 0.001$), Troponin T (3.3 ± 1.02 pg/ml) ($p < 0.05$), pro-BNP (32.5 ± 6.8 pg/ml) ($p < 0.001$) levels were found to be highest in this group. All three treatments were shown to ameliorate the toxic effect of doxorubicin in cardiac tissue with the best results in DXR + OX group. It is shown that tissue integrity, cellular apoptosis rate, QRS amplitude, plasma MDA, TNF-alpha, pentraxin-3, Troponin T, pro-BNP levels were closer to normal group in DXR + OX group than DXR + LIR and DXR + G-CSF groups. Among latter 2 groups, DXR + LIR group exerted better results than DXR + G-CSF group (Figs. 1, 2; Tables 1, 2, and 3).

Discussion

DXR has been used for the treatment of carcinomas and hematologic malignancies since discovery of the drug [12]. After started to be used extensively, physicians detected acute and chronic phase cardiotoxicity related to drug. In acute term, QT interval prolongation and arrhythmias were seem to be frequent. On the other hand with cumulative doses, they discovered that drug caused cardiomyopathy and heart failure with a dose-dependent manner especially in elderly. Chronic DXR-associated cardiotoxicity was found to develop about after 10 years of DXR usage [13]. Swain et al. [14] showed a cumulative dose of 400 mg/m² of DXR was related with a 5% increased risk of HF. This risk increased by 26% and 48% with the use of 550 and 700 mg/m² of DXR, respectively. In another study, at 500–550 mg/m² dose of DXR caused cardiomyopathy in 4% of patients, at the dose of 551–600 mg/m² this rate increased to %18 [15].

Fig. 1 Cardiac tissue histopathology H& E (**a, c, e, g, i**) and CASPASE-3 immunoeexpression in cardiomyocytes (**b, d, f, h, j**) ($\times 40$ magnification). **a** and **b** Normal cardiomyocytes, **c** and **d** DXR + saline group increased CASPASE 3 immunoeexpression (arrow) and disintegration of damaged cardiomyocytes (arrow), **e** and **f** DXR + LIR Group decreased CASPASE 3 immunoeexpression and cardiomyocytes showing regular sequence, **g** and **h** DXR + OX Group decreased CASPASE 3 immunoeexpression and cardiomyocytes showing regular sequence, **i** and **j** DXR + G-CSF Group decreased CASPASE 3 immunoeexpression

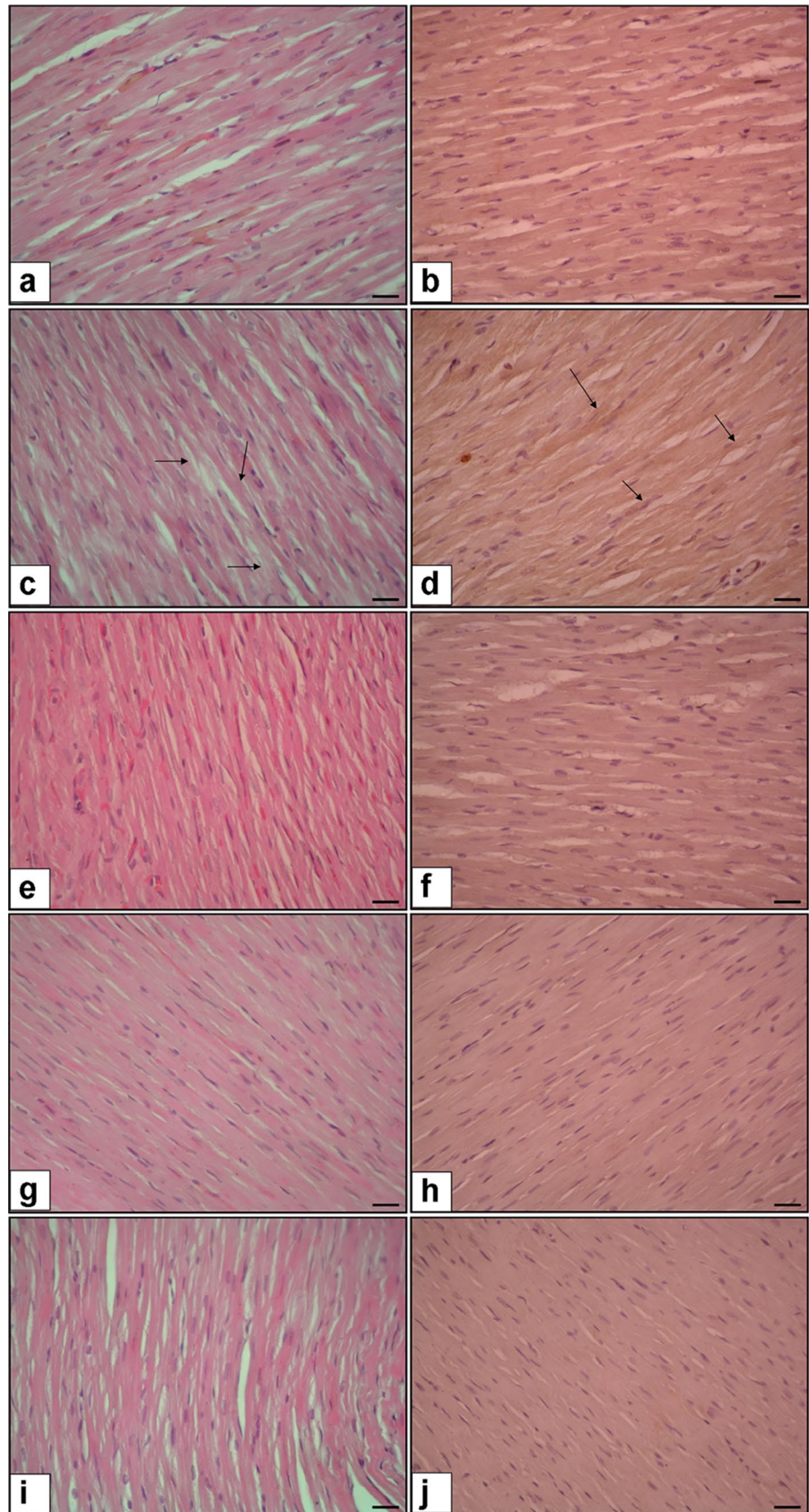


Fig. 2 ECG results of all groups. Derivation I ECG, **a** Normal Group, **b** DXR + saline group, **c** DXR + LIR group, **d** DXR + OX group, **e** DXR + G-CSF Group

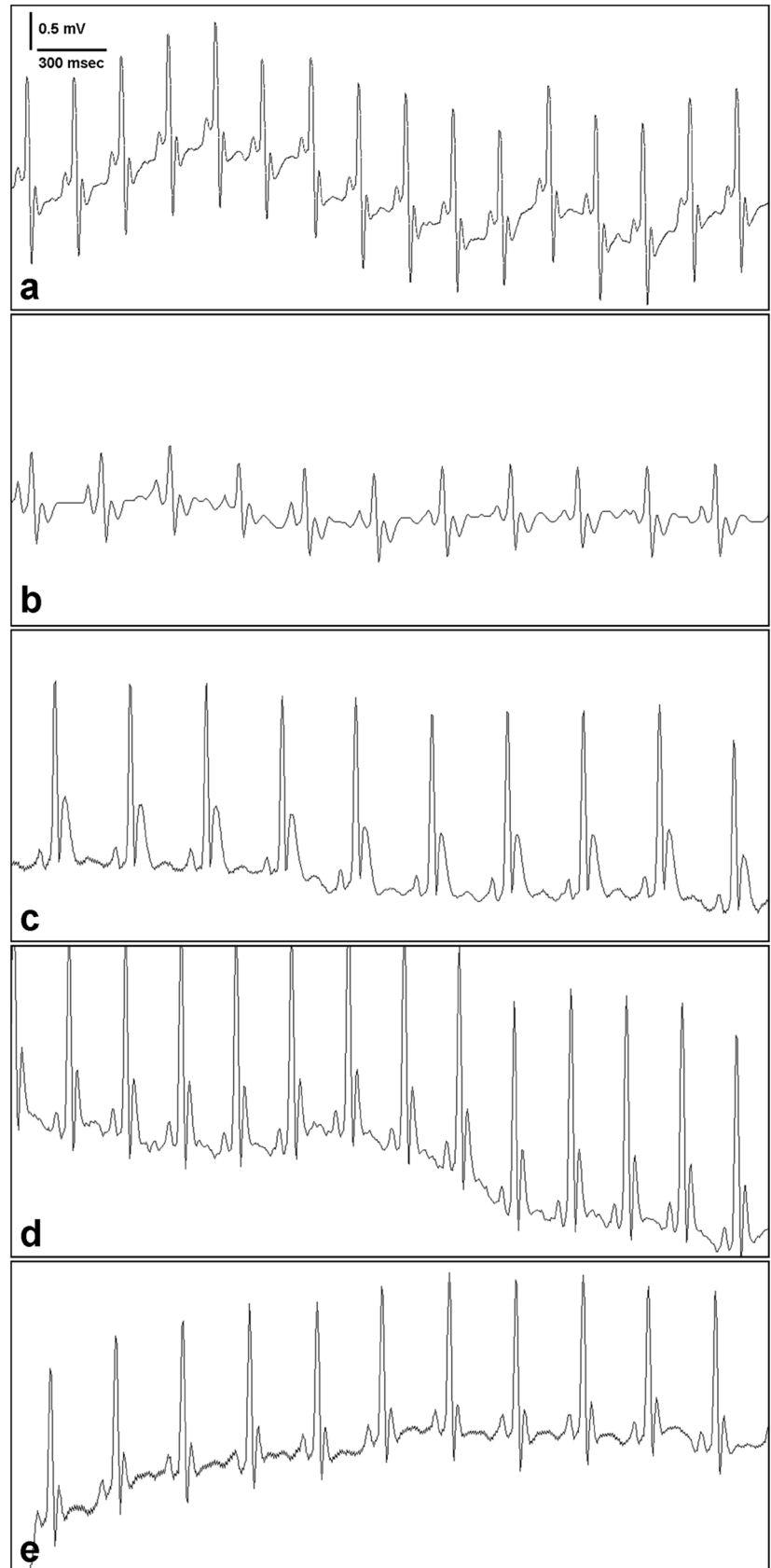


Table 1 CASPASE 3 immunoexpression results of all groups

	Normal group	DXR + saline group	DXR + LIR group	DXR + OX group	DXR + G-CSF group
CASPASE 3 immunoexpression	1.2 ± 0.1	14.6 ± 2.8*	7.2 ± 1.3 [#]	5.3 ± 0.9 [#]	8.8 ± 2.4 [#]

* $p < 0.000001$, DXR + Saline group compared with Normal group

[#] $p < 0.001$, DXR + LIR or DXR + OX or DXR + G-CSF group compared with DXR + Saline group

Table 2 ECG QRS amplitude results of all groups

	Normal group	DXR + Saline group	DXR + LIR group	DXR + OX group	DXR + G-CSF group
ECG QRS amplitude (mV)	0.24 ± 0.002	0.10 ± 0.004*	0.19 ± 0.003 [#]	0.21 ± 0.008 [#]	0.16 ± 0.007 [#]

* $p < 0.000001$, DXR + Saline group compared with Normal group

[#] $p < 0.00001$, DXR + LIR or DXR + OX or DXR + G-CSF group compared with DXR + Saline group

Table 3 Plasma MDA, TNF-alpha, Pentraxin-3, Troponin T and pro-BNP results of all groups

	Normal group	DXR + saline group	DXR + LIR group	DXR + OX group	DXR + G-CSF group
Plasma MDA (nm)	43.8 ± 6.01	328.1 ± 43.2**	215.7 ± 28.4 ^{##}	115.3 ± 19.8 ^{##}	278.8 ± 36.1 [#]
Plasma TNF-alpha (pg/ml)	17.6 ± 2.3	46.9 ± 4.1**	34.1 ± 5.1 ^{##}	26.6 ± 3.05 ^{##}	38.9 ± 6.3
Plasma Pentraxin-3 (ng/ml)	1.6 ± 0.08	4.7 ± 1.1**	3.3 ± 1.04 [#]	2.7 ± 0.9 ^{##}	4.1 ± 1.5
Pro-BNP (pg/ml)	4.15 ± 0.9	32.5 ± 6.8**	16.5 ± 4.2 [#]	11.1 ± 3.6 ^{##}	19.5 ± 7.2 ^{##}
Troponin T (pg/ml)	0.95 ± 1.04	3.3 ± 1.02*	2.1 ± 0.09 [#]	1.4 ± 0.08 ^{##}	2.04 ± 1.1 [#]

** $p < 0.001$, DXR + Saline group compared with Normal group

* $p < 0.05$, DXR + Saline group compared with Normal group

^{##} $p < 0.001$, DXR + LIR or DXR + OX or DXR + G-CSF group compared with DXR + Saline group

[#] $p < 0.01$, DXR + LIR or DXR + OX or DXR + G-CSF group compared with DXR + Saline group

DXR-induced cardiotoxicity has been investigated since discovery of the drug. Several mechanisms including increased oxidation of lipids [16], excessive production of reactive oxygen species (ROS) [17], increased inflammation, DXR-induced DNA damage [18] and apoptosis [19] have been suggested to enlighten this issue.

Among all of them, oxidative damage remains to be the pivotal mechanism. DXR accumulates in mitochondria and causes electron chain impairment which causes excessive production of superoxide radicals $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2). In the presence of Fe^{+2} , $O_2^{\cdot-}$ is converted to hydroxyl radical (OH^{\cdot}). These reactive oxygen species cause lipid peroxidation and DNA damage which leads cellular apoptosis and autophagy. While ROS damage the cell, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) try to defend organism by neutralizing these radicals with hydrogen element and producing H_2O [17]. We tried to evaluate oxidative stress by measuring plasma MDA level and inflammation by measuring plasma pentraxin-3 and TNF-alpha levels in our study.

A second mechanism may be the trigger of apoptosis by DXR treatment in cardiomyocytes. The extrinsic apoptosis

machinery is valid for rat cardiomyocytes, too. DXR treatment was shown to increase expression of death receptors like Fas/FasL as well as $TNF\alpha/TNFR1$ in rat cardiomyocytes by Wu et al. [20] and Niu et al. [21]. We tried to evaluate apoptosis rate in cardiac tissue by measuring microscopic CASPASE-3 immuno-expression level and define microscopic degree of tissue damage by scoring hemotoxylin–eosin staining slides. In addition, we recorded ECG form derivation I to assess clinical reflection of DXR-associated cardiotoxicity and measured QRS wave voltage amplitude as well as plasma Troponin T and pro-BNP levels which were proven to be reliable clinical indicators of cardiac tissue damage.

Oxytocin (OX) treatment caused the best results in our study. DXR + OX group has the most preserved tissue integrity, least immune expression level of CASPASE-3 (5.3 ± 0.9), ($p < 0.001$) the highest ECG QRS wave voltage amplitude (0.21 ± 0.008 mV) ($p < 0.00001$) least plasma MDA (115.3 ± 19.8 nm) ($p < 0.001$), TNF-alpha (26.6 ± 3.05 pg/ml) ($p < 0.001$), pentraxin-3 (2.7 ± 0.9 ng/ml) ($p < 0.001$), Troponin T (1.4 ± 0.08 pg/ml) ($p < 0.001$), pro-BNP (11.1 ± 3.6 pg/ml) ($p < 0.001$) levels among all three treatment groups. OX was found to have beneficial effects

on experimental Parkinson rat model [7], on experimental ovarian ischemia–reperfusion rat model [22], on experimental Cisplatin-induced neurotoxicity rat model [23], and on experimental diabetic polyneuropathy rat model [24]. In all these literature, it was suggested that OX exerts its cytoprotective effect via anti-inflammatory features. OX prevents neutrophil infiltration of damaged tissue, decreases TNF-alpha and IL-6 levels, reduces ROS levels and lipid peroxidation. In addition, OX attenuates NADPH-dependent superoxide production and has antioxidant effects on endothelium and vascular smooth muscle. Consistent with the previous literature, we found that OX treatment led decreased oxidative, apoptotic and inflammatory activity in DXR-induced cardiomyopathy rat model as well as provided better tissue integrity and better results in clinically relevant measures of ECG assessment, plasma Troponin T and pro-BNP levels.

Liraglutide (LIR) is shown to restore myocardial tissue integrity, reduce CASPASE-3 immunoeexpression (7.2 ± 1.3) ($p < 0.000001$), preserve QRS voltage amplitude (0.19 ± 0.003 mV) ($p < 0.000001$) reduce plasma MDA (215.7 ± 28.4 nm) ($p < 0.01$), TNF-alpha (34.1 ± 5.1 pg/ml) ($p < 0.01$), pentraxin-3 (3.3 ± 1.04 ng/ml) ($p < 0.01$), Troponin T (2.1 ± 0.09 pg/ml) ($p < 0.01$), pro-BNP (16.5 ± 4.2 pg/ml) ($p < 0.01$) levels when compared to DOX +saline group and all of the results were better than DOX + G-CSF group ($p < 0.01$). Liraglutide (LIR) was proven to have antioxidant effects in previous studies. It was shown that LIR has beneficial effects on non-alcoholic fatty liver disease animal model [25], LIR reduces SOD activity in macrophage derived foam cell culture model [4] and LIR attenuates DXR-induced elevation of proinflammatory cytokines like IL-1 and TNF-alpha. Li et al. [26] discovered that DXR treatment suppresses AMPK activity which leads p53 accumulation, apoptosis and elevated producing of ROS, eventually cell death. Abbas et al. [27] showed that LIR attenuates DXR-induced AMPK blockage thus ROS production and apoptosis of cell. They found that CASPASE-3 level induced by DXR can be reduced by LIR treatment in rats. Inhibition of apoptosis by reducing CASPASE-3 expression, increase in TNF-alpha and ROS levels, and increase in Bcl2 expression were suggested compounds of this beneficial mechanism in their paper.

In our study, LIR is proven to reduce proinflammatory cytokines as TNF-alpha, reduce lipid peroxidation products as MDA, reduce Troponin T, pentraxin-3 and pro-BNP levels and attenuates QRS voltage amplitude reduction and histopathologically shown cardiac tissue damage which are indicator of myocardium damage and heart failure, reduce CASPASE-3 level which is an indicator of apoptosis in DXR treated rats. Our findings are in consistent with previous findings. Furthermore, we showed pentraxin-3 level reduction which is a novel marker for inflammatory cardiac disease and provided ECG assessment data.

DXR + G-CSF group has the worst preserved tissue integrity, highest immune expression level of CASPASE-3 (8.8 ± 2.4) ($p < 0.001$) the least ECG QRS wave voltage amplitude (0.16 ± 0.007 mV) ($p < 0.00001$) the highest plasma MDA (278.8 ± 36.1 nm) ($p < 0.001$), TNF-alpha (38.9 ± 6.3 pg/ml) (statistically not meaningful), pentraxin-3 (4.1 ± 1.5 ng/ml) (statistically not meaningful), Troponin T (2.04 ± 1.1 pg/ml) ($p < 0.001$), pro-BNP (19.5 ± 7.2 pg/ml) ($p < 0.001$) levels among all three treatment groups.

G-CSF is extensively used to treat neutropenia after chemotherapy in hematologic malignancies. Recently, literature remarking cyto-protective and anti-inflammatory effects of G-CSF in some neurological diseases has been published [28].

G-CSF was shown to decrease infarct volume in an ischemic cerebral infarct rat model. Researchers suggested that G-CSF showed this effect by increasing stem cell production and migration to damaged tissue [8]. Studies of spine injury animal models were conducted to investigate G-CSF effect in damaged tissue and they revealed that G-CSF inhibited the apoptosis [9]. In a clinical study, patients with acute ischemic stroke treated with G-CSF for 12 months exhibited better results than patients who were not given G-CSF [29]. It was reported that G-CSF had anti-inflammatory effect by blocking synthesis of IL-1, TNF-alpha and had anti-apoptotic effect by increasing extracellular regulated kinase (ERK) signaling pathway in several other studies [30]. To date, there has been no another study which investigated the effect of G-CSF in DXR-induced cardiomyopathy animal model. Although results of DXR + G-CSF group were inferior when compared to DXR + OX and DXR + LIR groups, we found that G-CSF treatment in DXR cardiotoxicity revealed better results than DXR treatment alone and our findings were correlated with the previous literature.

Conclusion

Our findings suggest that with the best results in OX treatment group, all three agents including LIR and G-CSF attenuates tissue damage directly assessed by light microscopy and indirectly measured by plasma Troponin T and pro-BNP levels, inhibits apoptosis, preserves ECG QRS wave amplitude, decreases inflammation and lipid peroxidation indirectly measured by plasma MDA, TNF-alpha and pentraxin-3 levels in the rat model of DXR-induced cardiomyopathy. Although our study did not specifically define the exact mechanism of action of selected drugs, we showed their beneficial effect directly in cardiac tissue and indirectly proved their anti-inflammatory effect by measuring biochemical markers of inflammation and heart failure. Previous findings in the literature supports these findings in different sets of animal disease models such as diabetes,

Parkinson, neuropathy. Clinical studies to assess effects of these drugs in mentioned diseases still remain to be conducted for more accurate data.

Compliance with Ethical Standards

Conflict of interest The authors did not declare any conflict of interest.

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