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Assessment of the cardioprotective effect of liraglutide on methotrexate induced cardiac dysfunction through suppression of inflammation and enhancement of angiogenesis in rats

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Abstract. – OBJECTIVE: Methotrexate (MTX) is one of the most commonly used anti-cancer drugs for various types of neoplasms. It is associated with multiple cytotoxic effects including nephrotoxicity, hepatotoxicity and cardiotoxicity. Liraglutide (LIR) is a potent anti-diabetic drug and also has antioxidant and anti-inflammatory properties. In this study, we tried to investigate the protective effect of LIR on MTX induced cardiotoxicity and to identify the molecular mechanisms for this protection.

MATERIALS AND METHODS: Rats were divided into 4 groups, including control group, LIR group, MTX group and LIR + MTX group. ECG was measured then blood samples were taken, and hearts were excised for biochemical and histological investigations.

RESULTS: MTX group exhibited a mild non-significant irregular bradycardia, an increase of CK-MB besides a decrease of total antioxidant capacity. MTX administration also resulted in downregulation of vascular endothelial growth factor (VEGF), while caused upregulation of interleukin 1 beta (IL-1B) and interleukin 6 (IL-6) in comparison to the control group. Also, MTX group showed histological abnormalities besides negative VEGF and positive iNOS as detected by immunohistochemical staining compared to the control group. LIR administration could reverse these results.

CONCLUSIONS: LIR prevented MTX induced cardiotoxicity through its antioxidant and anti-inflammatory properties.

Key Words:
Methotrexate, Liraglutide, Cardiotoxicity, Vascular endothelial growth factor, Interleukin 1 beta, Interleukin 6.

Introduction

Cardiotoxicity is defined as cardiac muscle dysfunction, which may proceed to heart failure (HF). The degree of cardiotoxicity is determined according to represented symptoms or a decrease in the ejection fraction. Many different types of cardiotoxicities have been documented[1].

One of the most important causes of cardiotoxicity results from chemotherapy[2], which considered as a huge problem because toxicity may consume long duration for accumulation of the drug or its metabolites to be manifested[3]. The chemotherapeutic drugs that cause cardiotoxicity are anthracyclines (ANTs), fluorouracil and paclitaxel. Hypoxia and oxidative stress are the main mechanisms for the establishment of cardiotoxicity[4].

Many factors affect the progression of cardiac toxicity including the concentrations of drugs, genetic disorders and underlying heart diseases[5]. The effects of chemotherapeutic agents on the cardiovascular system cause alterations in myocardial structure and function even may lead to heart failure, arrhythmias, hypertension, myocardial ischemia, pericardial disease and thromboembolic disease[1]. ANTs may lead to irreversible cardiovascular toxicity through the formation of reactive nitrogen species (RNS) and reactive oxygen species (ROS), while the induced toxicity by biological drugs is usually reversible[6].

Methotrexate (MTX) is a common anti-cancer drug used for the treatment of different malignancies like lymphoma. Interestingly, MTX in low

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doses has anti-inflammatory and immunosuppressive action so can be used in the treatment of multiple autoimmune diseases. The cytotoxic effects of MTX do not only destroy tumor cells but also influence the vital organs, such as the heart[7]. Other serious side effects of MTX included renal affection, which was represented by a reduction of creatinine clearance, hematuria and cystitis[8]. On the other hand, respiratory system disturbances are present in the form of acute interstitial pneumonitis, pleural effusion and interstitial fibrosis[9].

Liraglutide (LIR) is a long-acting glucagon-like peptide 1 (GLP1) receptor agonist that is similar to human GLP1 by about 97% sequence homology. LIR was documented by the FDA for the treatment of hyperglycemia in type 2 diabetic patients. The protective role of LIR on the hepatic tissues of diabetic patients associated with obesity was confirmed. Many studies have revealed that LIR can increase neurogenesis, improve cognitive function, and reduce amyloid plaque deposition found in Alzheimer's disease[10,11].

LIR has antiinflammatory and antioxidant activities. LIR exhibits anti-inflammatory effects on different organs through decreasing inflammatory cytokines production in the tissues[11]. Also, GLP1 was used for treatment of chronic inflammatory diseases such as atherosclerosis and neurodegenerative disorders[12]. Recent studies were performed to demonstrate the cardiovascular safety and efficacy of LIR[13].

The aim of the current study is to detect the molecular mechanisms of cardiotoxicity induced by the chemotherapeutic drug, methotrexate and to assess the protective effects of liraglutide on this toxicity.

Materials and Method

Drugs and Chemicals

Methotrexate (50 mg/5 mL injectable solution) was obtained from Mina pharm Pharmaceuticals (Cairo, Egypt).

Liraglutide was bought from Novo Nordisk S.p.A. (Rome, Italy).

Animals and Housing

Thirty-two adult male albino rats, their weights were ranging from 175 to 220 g. They were housed in standard plastic cages in a room with regulated temperature (20-22°C), a dark-light period of 12:12 h and fed a balanced diet and tap water. Rats were adapted to laboratory conditions one week before the beginning of the experiment. All animals' procedures were according to the Institutional Ethics Committee for animal research of Fayoum University (Fayoum, Egypt).

Experimental Design

Animals were randomly divided into four groups:

Group 1: CTRL-vehicle, served as negative control, received only saline subcutaneously (S.C.) for 10 days.

Group 2: CTRL-LIR, 250 µL of liraglutide solution was diluted in 5 mL of saline solution (0.9% NaCl) to obtain a final solution of 300 µg/mL and given (S.C.) at a dose of 300 µg/kg/day for 10 days[10].

Group 3: MTX, rats received only methotrexate intraperitoneally (I.P.) as a single dose of 20 mg/kg on the tenth day[14]. Acute administration of Methotrexate was used to induce cardiotoxicity.

Group 4: MTX-LIR, rats were given both MTX and LIR at the same doses that previously mentioned.

ECG Recording

At the end of the experiment, ECG was performed for rats using Electrocardiogram (KENZ ECG 106, Nagoya, Japan) for assessment of heart rate (HR).

Sample Collection

Three days after MTX injection, rats were anesthetized using I.P injection of thiopental sodium 50 mg/kg then blood was taken out from the retro-orbital plexus of each. Centrifugation at 3000 rpm for 15 min at 4°C was used to separate serum. The collected serum was stored at -80°C until analysis. Rats were sacrificed by cervical dislocation and hearts were excised. Portions of dissected cardiac tissues were placed in 10% buffered formalin for histopathological investigation, other parts were used for biochemical investigation.

Biochemical Analyses

Serum concentrations of creatine kinase isoenzyme (CK-MB) in different groups were analyzed using ELISA technique (SunLong Biotech Co, LTD, China).

Oxidative Stress Detection

Colorimetric determination of NO and TAC was done using commercially available kits ob-

tained from Bio diagnostic Co. (Giza, Egypt). The cardiac tissues were perfused with phosphate-buffered saline (PBS) following dissection. Tissues were added to cold buffer, ground into homogenates and centrifuged at 4000 rpm at 4°C for 15 min, then, the supernatant was gathered for the measurement of NO. The levels of total antioxidant capacity (TAC) were assayed in the serum.

Quantitative Real Time-PCR (qRT-PCR) Investigation

Total RNA was isolated from cardiac tissues homogenates using tissue extraction kit (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was confirmed using NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Extracted RNA was used for reverse transcription-PCR (RT-PCR) for cDNA production using the QuantiTect® Reverse Transcription Kit as detected in the manufacturer's protocol (Qiagen GmbH, Hilden, Germany) in a final reaction volume of 20 µl. Real time RT-PCR reactions were carried out using gene expression assays for interlukin-1B (IL-1B), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) and B-actin (Biotez Berlin-Buch GmbH, Berlin, Germany). The primer sequences utilized were as follows: for IL-1B: forward primer 5′-CCA GGA TGA GGA CCC AAG CA-3′, reverse primer: 5′-TCC CGA CCA TTG CTG TTT CC-3′; for IL-6: forward primer 5′-ATG AAG TTT CTC TCC GCA AGA GAC TTC CAG CCA-3′, reverse primer: 5′-CTA GGT TTG CCG AGT AGA CCT CAT CAT AGT GAC C-3′; for VEGF: forward primer 5′-ACC TCC ACC ATG CCA AGT-3′, reverse primer 5′-TTG GTC TGC ATT CAC ATC TG-3′; and for B-actin: forward primer 5′-GAT ATC GCT GCG CTC GTC-3′, reverse primer: 5′-TGG GGT ACT TCA GGG TCA GG-3′. Real-time RT-PCR was achieved in a 25-µl reaction volume consisting of QuantiTect SYBR Green PCR Master Mix, 2.5 µl of each primer and 5 µl of cDNA. Thermal cycling conditions were used, including a pre-amplification step of 95°C for 15 min, followed by amplification for 40 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s. Relative expression of the studied genes mRNA was measured using the threshold cycle method ($2^{-\Delta\Delta Ct}$)[15]. All values were demonstrated as the ratio of the specific genes to B-actin which was used as an internal control gene where the relative value for the control group was detected as one.

Histopathological Examination

Tissue washing was done by tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and fixed in paraffin at 56°C in a hot air oven for 24 h. Sections were embedded in paraffin and sliced into 4 μm thick sections by a sledge microtome. The tissue sections were collected on glass slides, deparaffinized, stained with hematoxylin & eosin stain (H&E) then examined by light microscope.

Quantitative Immunohistochemical Analysis of VEGF and NO

Four microns' thick paraffin embedded tissue sections were prepared. Immunohistochemical analysis was performed according to the manufacturer's protocol[16]. Depraffinized retrieved tissue sections were treated by 0.3% H2O2 for 20 min. After that, heart samples were incubated with anti-iNOS (PA1-036) (1:20) (Thermo Fisher Scientific Waltham, USA) and Anti-VEGF (ab1316) (1:200) (Abcam, Waltham, USA) at 4°C overnight. Tissue sections washed out by PBS followed by incubation with secondary antibody HRP Envision kit (DAKO) 20 min; washed out and incubated with diaminobenzidine (DAB) for 15 min. Tissue sections washed by PBS then counter stained with hematoxylin, dehydrated and cleared in xylene then cover slipped for microscopic examination.

Morphometric Study

The morphometric data were collected using the image analyzer computer system (Leica Qwin 500, England). The area % of INOS and VEGF immune expression were measured in 10 high power non- overlapping fields in each specimen using binary mode.

Statistical Analysis

The data was represented as mean ± standard deviation (mean ± SD). The Analysis of Variance (ANOVA) and the post hoc test were be used to compare groups. *p*-value below 0.05 is assumed to be statistically significant.

Results

LIR Improved Changes of Body Weight and Abnormalities of ECG

Table I illustrated that at the beginning of the experiment, no difference in the body weight

was noticed among all groups. By the end of the study, rats received LIR (group II) exhibited mild non-significant reduction of body weight as compared to control rats (group I), while MTX group (group III) showed a significant reduction of weight when compared to control group. Combination of LIR with MTX (group IV) decreased the weight with no significant difference with the control group (Table I).

Regarding the percentage of body weight Change, a significant decrease for each of all treated groups (LIR, MTX and MTX-LIR) was detected in comparison to control rats ($p \leq 0.0001$, $p \leq 0.0001$ and $p=0.001$ respectively (Table I).

As observed in Table I, the treatment with LIR only (group II) showed an increase of heart rate as compared to control group (group I). Rats administrated with MTX (group III) revealed non-significant irregular bradycardia when compared to other groups. Group of combined intakes of LIR and MTX (group IV) showed non-significant increase of heart rate and correction of the irregularities that produced as a result of MTX administration (Table I).

LIR Decreased MTX Induced Oxidative Stress and Cardiotoxic Markers

The cardiac levels of the oxidative stress marker NO were significantly increased in group III compared to group I. In addition, group III had significantly lower serum levels of TAC than group I. On the other hand, group IV showed significant lower levels of NO and significant higher levels of TAC when compared to group III. Interestingly, the levels of cardiac NO were significantly higher in group IV compared to group I ($p<0.0001$), while the serum TAC show no significant difference between two groups ($p=0.162$). These results indicated that LR can attenuate MTX induced oxidative stress. Moreover, administration of LR only (group II) showed lower cardiac NO levels and higher serum TAC levels with no significant difference when compared to controls ($p=0.429$ and 0.992 respectively) (Table I).

MTX injected rats (group III) showed significantly elevated serum levels of the cardiac damage marker CK-mb compared to the control group (Table I). In contrast, LIR- MTX treated rats (group IV) detected a significant reduction in CK-mb levels compared to group III, but levels were significantly higher than in group I. These results indicate that LIR can reduce the level of CK-mb induced by MTX cardiotoxicity (Table I).

LIR Reduced MTX Triggered Inflammation

The cardiac inflammatory markers IL-1B and IL-6 were significantly upregulated in MTX intoxicated rats (group II) compared to the control group (not demonstrated in the table as the gene expression levels are equal to 1) (Table II). In contrast, combined administration of LIR and MTX (group III) showed a significant down regulation for these markers in the heart tissues compared to MTX group (group II).

Furthermore, MTX treated group showed significant down regulation of VEGF compared to control group. However, pretreatment with LIR in MTX intoxicated rats (group III) significantly upregulated the gene expression of VEGF when compared to MTX group (group II). Additionally, combined treatment of LIR with MTX (group III) exhibited no significant difference with control group regarding VEGF expression levels, while there was a significant difference regarding IL-1B and IL-6. Interestingly, it was determined a significant increase in the expression levels of VEGF in LIR treated group (group I) when compared to controls ($p=0.002$). Thus, LIR could decrease MTX triggered inflammation and improved angiogenesis (Table II).

Table I. The effect of liraglutide and methotrexate on biochemical markers and body weight change in MTX induced cardiotoxicity in rats.

	Control group	LR group	MTX group	MTX+LR group
NO (µmol/gm tissue)	60.92±13.444	55.38±13.31^{c}	267.5±54.7^{ab}	159.19±40.48^{abc}
TAC (mM/L)	1.76.±.334	1.96±.36^{c}	0.59±.094^{ab}	1.43±.218^{bc}
CK-mb (ng/ml)	0.845±0.179	0.585±0.15^{c}	7.69±1.01^{ab}	4.6299±1.182^{abc}
Body weight change (%)	4.07±1.37	-6.46-±4.33^{a}	-8.83-±2.92^{a}	-5.57-±5.40^{a}

The results are expressed as mean ±SD; n= 8 each. $^{a}p <$ as compared to the control group, $^{b}p < 0.05$ as compared to the LR group, $^{c}p< 0.05$ as compared to the MTX group. NO, nitric oxide; TAC, total antioxidant capacity; CK-mb, creatine kinase-mb. $p < 0.05$ is considered a significant value.

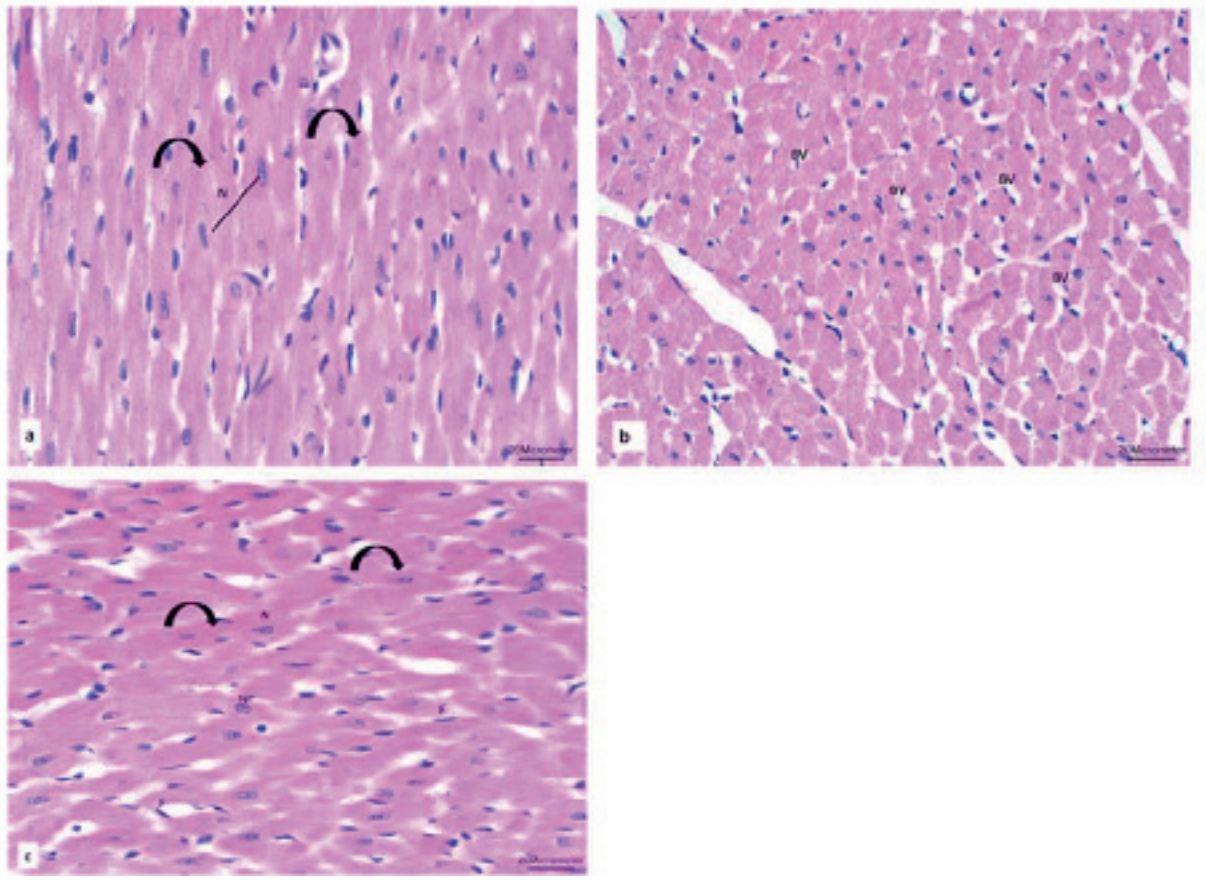

Figure 1. A photomicrograph of a rat myocardium from control group (group I) **(a)** showing normal architecture of myocardium; branching and anastomosing cardiac muscle fibers (curved arrow) running in different directions with central oval vesicular nuclei (N), acidophilic sarcoplasm and few spindle shaped fibroblast (F) in the connective tissue in-between muscle fibers. **(b)** showing transverse section of cardiac muscle with many small thin-walled blood vessels (BV) in-between fibers. Liraglutide group (group II) **(c):** showing normal architecture of myocardium. (H&E X400).

Hematoxylin and Eosin Stain

Control and LIR groups (groups I and II) showed normal architecture of myocardium; branching and anastomosing cardiac muscle fibers running in different directions with central oval vesicular nuclei, acidophilic sarcoplasm, few spindle shaped fibroblast in the connective tissue in between muscle fibers (Figure 1 a, c) and transverse section of cardiac muscle with many small thin-walled blood vessels in-between fibers (Figure 1 b**)**.

In MTX intoxicated group (group III); some pale cardiac muscle cells appeared with nuclear

Table II. The effect of liraglutide, methotrexate and combined administration of liraglutide with methotrexate on the gene expression levels of IL-1B, IL-6 and VEGF in cardiac tissue of methotrexate induced cardiotoxicity in rats.

	LIR group	MTX group	MTX+LIR group
IL-1B	1.08 ± 0.31^{c}	4.37 ± 1.31^{ab}	2.24 ± 0.62^{abc}
IL-6	0.96 ± 0.43^{c}	3.85 ± 0.85^{ab}	2.28 ± 0.49^{abc}
VEGF	2.44 ± 0.55^{ac}	0.34 ± 0.15^{ab}	0.82 ± 0.19^{bc}

Results were expressed as mean ± SD, (n=8). Expression levels in the control group are equivalent to 1. $^{a}p< 0.05$ as compared to the control group. $^{b}p < 0.05$ as compared to the CTRL-LIR group, $p^{c}< 0.05$ as compared to the MTX group. $p < 0.05$ is considered a significant value.

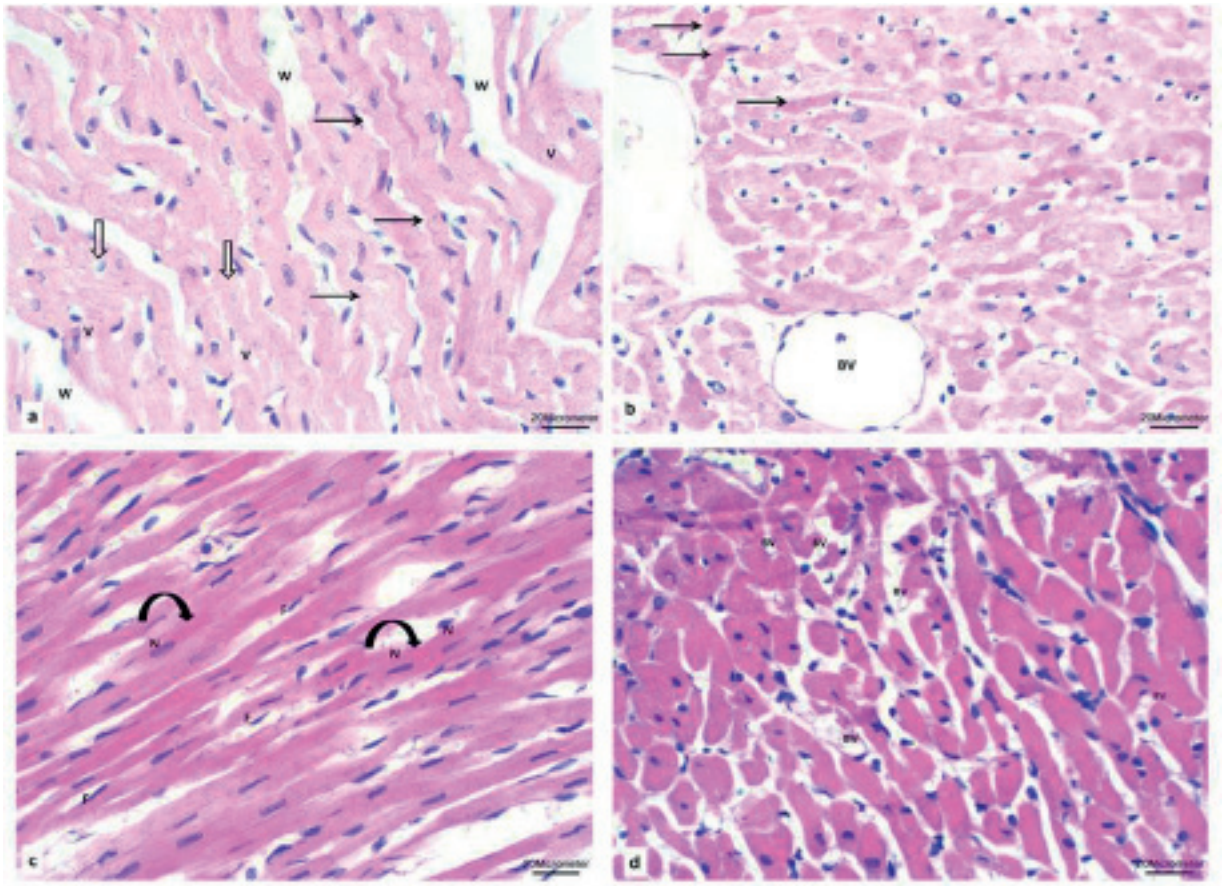

Figure 2. A photomicrograph of sections of rat heart from methotrexate group (group III) **(a)** showing pale cardiac muscle cells with nuclear ghosts (thick arrow) and vacuolation (V) in sarcoplasm, waviness of muscle fibers (thin arrow) and wide interstitial space between fibers (W). **(b)** showing darkly eosinophilic cardiac muscle cells with small pyknotic nuclei (thin arrow) and dilated blood vessel (BV). Liraglutide and methotrexate group (group IV) **(c)** showing normal arrangement of cardiac muscle fibers (curved arrows) with central oval vesicular nuclei (N) and acidophilic sarcoplasm separated with little interstitial spaces containing few spindle shaped fibroblast (F). **(d)** showing transverse section of cardiac muscle with many small thin-walled blood vessels (BV) in-between fibers. (H&E X400).

ghosts and vacuolation in sarcoplasm, waviness of muscle fibers, wide interstitial space between fibers (Figure 2 a), dark eosinophilic cardiac muscle cells with small pyknotic nuclei and dilated blood vessel (Figure 2 b).

In LIR and MTX group (group IV); normal arrangement of cardiac muscle fibers with central oval vesicular nuclei, acidophilic sarcoplasm separated with little interstitial spaces containing few spindles shaped fibroblast (Figure 2 c) and transverse section of cardiac muscle with many small thin-walled blood vessels in-between fibers were detected (Figure 2 d).

INOS Immunostaining

Control and LIR groups showed negative immunoexpression of INOS in cardiac muscle fibers (Figure 3 a, b) (Table III). However, MTX group (group III) showed positive immunoexpression of INOS in curved cardiac muscle fibers (Figure 3 c) (Table III). LIR and MTX group (group IV) showed negative immunoexpression of INOS in cardiac muscle fibers and weak positive immunoexpression of INOS in some cardiac muscle fibers (Figure 3 d) (Table III).

VEGF Immunostaining

Control and LIR groups showed positive immunoexpression of VEGF in endothelial cells of many blood vessels (Figure 4 a, b) (Table III). MTX group (group III) showed negative immunoexpression of VEGF in endothelial cells of blood vessels (Figure 4 c) (Table III). LIR and MTX group (group IV) showed positive immunoexpression of VEGF in endothelial cells of blood vessels (Figure 4 d) (Table III).

Table III. The area % of INOS and VEGF obtained from the different groups of the examined animals.

	Control group	LIR group	MTX group	MTX+LIR group
Area% of INOS	0.31±0.26	0.35±0.21	19.55±2.65[ab]	1.27±0.51[c]
Area% of VEGF	30.68±1.88	48.71±4.77[a]	6.64±1.87[ab]	23.59±0.92[abc]

Results were expressed as mean ± SD, (n=8). [a]$p < 0.05$ as compared to the control group. [b]$p < 0.05$ as compared to the CTRL-LIR group, [c]$p < 0.05$ as compared to the MTX group. $p < 0.05$ is considered a significant value.

Discussion

In the current study, methotrexate group showed non-significant decrease of heart rate with irregular rhythm related to other groups. Administration of LIR reversed these results. Investigations of ECG changes associated with methotrexate are limited. Methotrexate was reported to induce ventricular arrhythmia[17,18]. Meanwhile other anti-cancer drugs were documented to affect heart rate. Doxorubicin exhibited controversial effects as reported by Lončar-Turukalo et al[19] who detected variable alternations in heart rate were associated with doxorubicin treatment. Warpe et al[20] found that rats received doxorubicin acquired bradycardia. Moreover, other anti-cancer drugs were demonstrated to cause sinus bradycardia[21] such as Paclitaxel[22], Thalidomide[23] and Ceritinib[24].

On the other hand, LIR was found to increase heart rate in type 2 diabetic patients as a compensatory

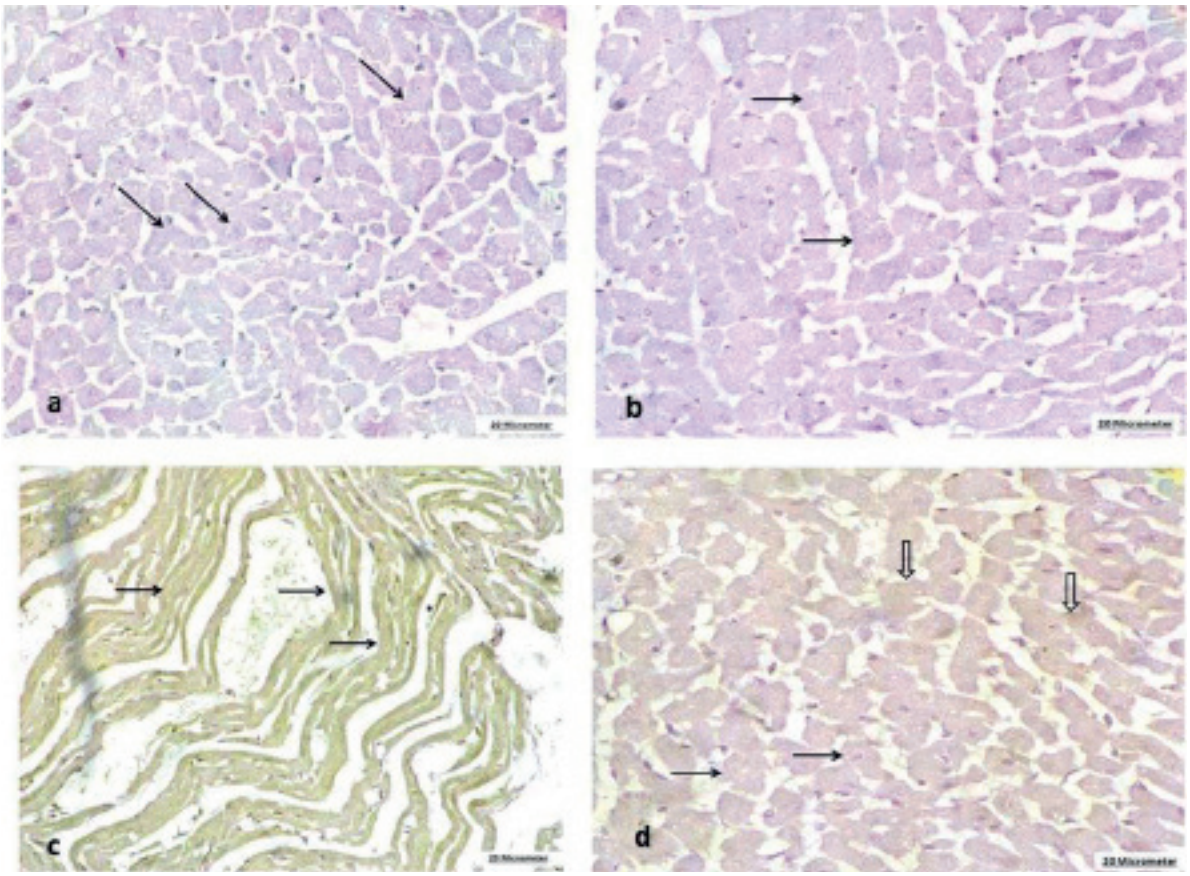

Figure 3. A photomicrograph of sections of rat heart stained with INOS immunostain **(a):** control group (group I) showing negative immunoexpression in cardiac muscle fibers (thin arrows). **(b):** liraglutide group (group II) showing negative immunoexpression in fibers (thin arrows). **(c):** methotrexate group (group III) showing positive immunoexpression in curved cardiac muscle fibers (thin arrows). **(d):** liraglutide and methotrexate group (group IV) showing negative immunoexpression in cardiac muscle fibers (thin arrows) and weak positive immunoexpression in some fibers (thick arrows). (INOS ×400).

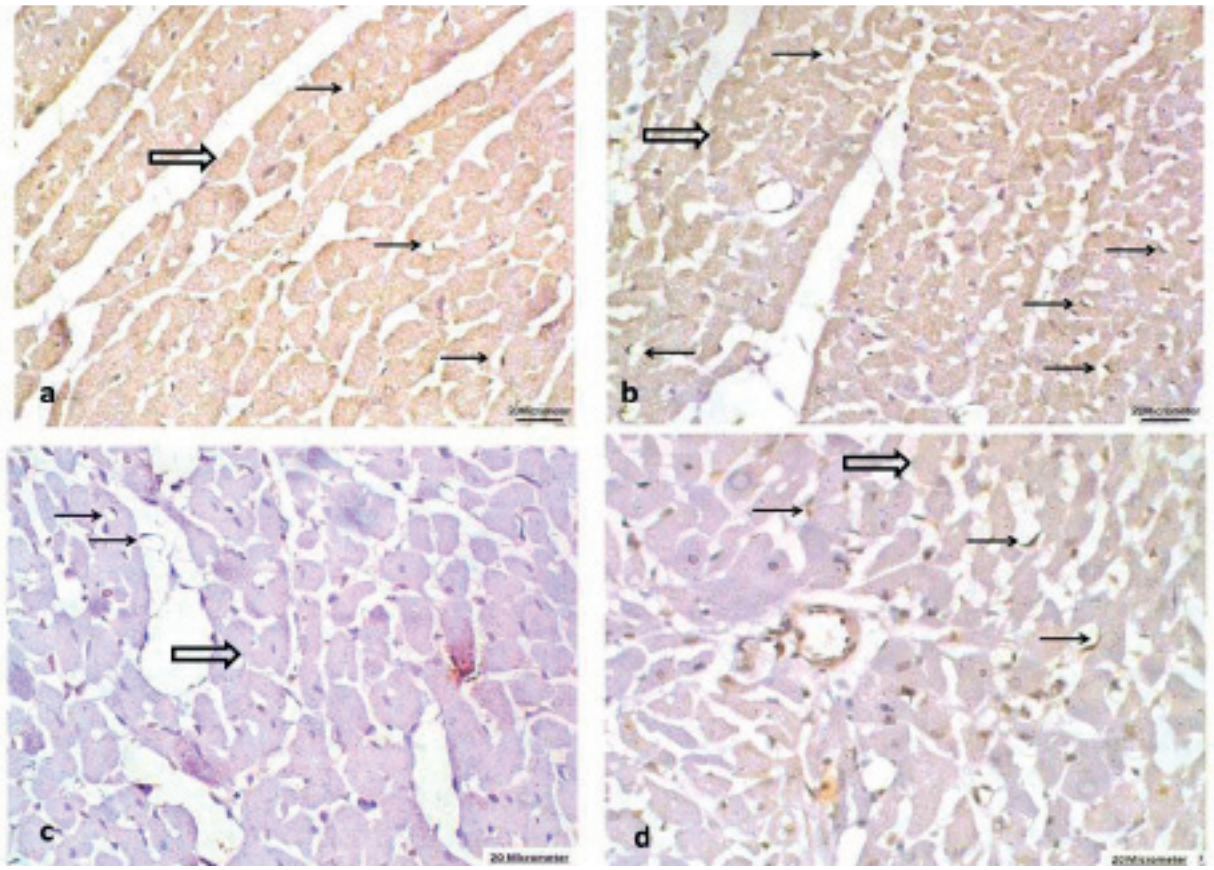

Figure 4. A photomicrograph of sections of rat heart stained with VEGF immunostain **(a):** control group (group I) showing positive immunoexpression in endothelial cells of many blood vessels (arrows). **(b):** liraglutide group (group II) showing positive immunoexpression in endothelial cells of many blood vessels (arrows). **(c):** methotrexate group (group III) showing negative immunoexpression in endothelial cells of blood vessels (arrows). **(d):** liraglutide and methotrexate group (group IV) showing positive immunoexpression in endothelial cells of blood vessels (arrows). (VEGF ×400).

mechanism against cardiovascular complications[25,26]. Hara et al[27] explored that the increase of heart rate associated with LIR therapy may be concerning with the impairment of parasympathetic activity and increased the activity of sympathetic functions.

In the current study, methotrexate developed cardiac toxicity as evidenced by histopathological studies. Moreover, MTX treated rats established cardiac damage leading to a significant elevation of cardiac enzyme CK-MB and excessive production of oxidative stress in the form of a decrease of TAC and an increase of NO when compared to control group. On the cellular level, MTX up regulated IL-1B and IL-6 and reduced VEGF expression. LIR pretreatment conversely contracted these effects and led to protection for the heart through increase of TAC and decreased CK-MB and NO levels. Moreover, down regulation of IL-1B and IL-6 and upregulation of VEGF expression levels were detected.

Similar to the findings of this study, Al-Taher et al[28] revealed the protective effect of Paeonol against cardiac toxicity induced by MTX in rats *via* promotion of oxidative cardiac tissue damage as a result of disturbance in oxidant/antioxidant balance.

A previous study[29] was investigating the role of thymoquinone on MTX induced intestinal damage. It concluded that MTX caused nitrosative stress as it increased NO level and upregulated inducible NO synthase (iNOS) as detected by immune histochemical staining. NO can activate NF-κB that is responsible for regulation of inflammatory cytokines, such as IL-1B, IL-6, IL-8 and TNF-α[28,30]. MTX contributed in development of cerebral and intestinal toxicity through enhancement of oxidative stress represented by increasing the oxidative stress markers as MDA[31,32]. Moreover, MTX could inhibit antioxidant transcription factor nuclear factor-erythroid 2-related factor 2 (NRF2) which is involved in the formation of endogenous antioxidants like SOD[14,33].

On the other hand, MTX was observed to exhibit antioxidant characters in some autoimmune diseases as rheumatoid arthritis[34]. MTX significantly decreased the elevated serum NO in collagen induced arthritis in rats[35]. This may be explained as administration of MTX in these conditions, was in small doses for short durations. While using it for oncology indications, other mechanisms were reported including interactions with adenosine pathways and generation of ROS[36,37].

In this study, MTX caused upregulation of IL-1B and IL-6, key inflammatory cytokines that play an imperative role in cardiac remodeling[38]. These results are consistent with the findings of other studies that stated that MTX induced renal, hepatic and cardiac injury in mice was associated with a significant increase in the levels of IL-1B and IL-6 simultaneously with the increase of iNOS and NO[33,39]. MTX was found to cause upregulation and production of IL-1, IL-6 and TNF-α through the NF-κB pathway[40].

Administration of LIR prior to MTX caused elevation of TAC and reduction of CK-MB and NO levels in addition to downregulation of IL-1B and IL-6 compared to methotrexate group. Comparable to these findings, Zhu and his colleagues reported the ability of LIR to prevent lipotoxicity in hepatic cells by increasing TAC level[41]. LIR increased SOD activity while decreased MDA, CK-MB and IL-6 with attenuation of inflammation and necrosis[38].

LIR increases survival kinases as extracellular signal-regulated kinase ½ (ERK1/2) and Protein kinase B (AKT) besides down regulation of stress-activated kinases C-Jun N-terminal kinase (JNK) and P38 that leads to inhibition of NF-κB[42,43]. Antioxidant properties of LIR were confirmed through upregulation of Nrf2 that responsible for transcription of antioxidant agents as SOD and glutathione. Furthermore, LIR contributes to decreasing of MDA level[44].

LIR increased the expression of anti-inflammatory markers as IL-10 in the hippocampi of rats' males[45]. IL-10 receptor is involved in different signals through inhibition of gene expression of different inflammatory cytokines like TNF-α, IL-1B, IL-6, IL-8, IL-12 and IL-23[46] and extends to a downregulation of the major histocompatibility complex class II (MHC-II)[47]. Also, LIR attenuated Non-Alcoholic Fatty Liver associated with diabetes through down regulation of IL-1B and NF-κb[48].

The current results showed that MTX induced cardiac tissue damage revealed by histopathological changes. Some dark eosinophilic cardiac muscle cells with small pyknotic nuclei were observed. Other cells appeared pale with nuclear ghosts or without nuclei in addition to vacuolation in sarcoplasm. Wide interstitial space between fibers and dilated blood vessels were detected. To great extent, LIR normalized these findings.

Protective effects of LIR in the present work is in line with the results of Abbas et al[38] who found that LIR prevented doxorubicin associated myocardial injury. Consistently with the present study, LIR could attenuate Gefitinib induced cardiotoxicity as it reversed hyperchromasia in the myocardial fibers nuclei and decreased ventricular hypertrophy[43].

There is a strong association between insulin resistance and chronic heart failure[49]. GLP1 exerts cardio protective effects as it improves insulin sensitivity and activates c-AMP mediated signaling pathways in cardiac muscle cells[50]. LIR activates AKT and increases AMP-activated protein kinase (AMPK) activity so antagonize the development of insulin resistance[38].

Vascular homeostasis is a balanced state between endothelial cells and vascular smooth muscle cells in the vascular wall. VEGF, a bioactive substance that is essential to maintain this balance[51]. In the present study, MTX group showed down regulation of VEGF compared to control group, while it was up regulated by LIR. A high link was approved between VEGF inhibitors and development of cardiotoxicity[52] which may be related to the development of perfusion contraction mismatching[53,54].

To our knowledge, the link between methotrexate induced cardiotoxicity and VEGF expression was not expressed before, but previous studies[55,56] proved that methotrexate performed its therapeutic activity against autoimmune diseases through downregulation of VEGF such as rheumatoid arthritis and psoriasis.

GLP1 was detected to stimulate the process of angiogenesis through up regulation of VEGF[57]. LIR increased the expression of VEGF in a model of cerebral ischemia[58]. LIR reduced the size of myocardial infarction and significantly increased the expression of VEGF and hypoxia-inducible factor 1α (HIF1α) through upregulation of endoplasmic reticulum position protein homologue 2 (PCNY2), that contributed in angiogenesis[59]. Moreover, LIR improved angiogenesis and enhanced revascularization in transplanted islets via up regulation of mammalian target of rapamycin pathway that was reported to increase HIF1α levels and thus increased VEGF[60].

Consequently, in the current study, the cardioprotective effect of LIR against methotrexate induced cardiotoxicity appears to be secondary to

its antioxidant and anti-inflammatory activities in addition to stimulation of angiogenesis through up regulation of VEGF.

The study of MTX induced cardiotoxicity has not received as much attention as the other side effects resulting from methotrexate administration. In this work, we investigated many novel points related to MTX cardiotoxicity that were not clearly analyzed in previous studies. These points included ECG abnormalities associated with MTX administration and involvement of VEGF expression in the development of MTX cardiotoxicity.

Conclusions

Liraglutide, a potent anti-diabetic drug improved cardiac disorders associated with MTX. Liraglutide improved ECG results and significantly decreased oxidative stress and inflammation through down regulation of IL-1B and IL-6. In addition, liraglutide stimulated angiogenesis through up regulation of VEGF. Along with reducing polypharmacy, this study aims to that liraglutide which acts as a conventional anti-diabetic drug could be repositioned to improve cardiotoxicity due to its antioxidant anti-inflammatory activity.

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Conflicts of interest
The authors declare that they have no conflict of interests.

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Data Availability
Data available on request due to privacy.

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