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Modulation of cardio-metabolic disorders by Tocilizumab in rats with fructose-induced metabolic syndrome: Role of cardiac NLRP3 Inflammasome and TIMP1

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Abstract:

Introduction: Insufficient regulation of NLRP3 inflammasome and TIMP1 has a role in the pathogenesis of CVD. Moreover, tocilizumab has a cardio-metabolic protective effect mainly through improved metabolic indices, IL6, alongside cardiac TNF α , NLRP3, and TIMP1 activity.

Aim of the study: The study aims to examine the effect of IL6 receptor blocker; tocilizumab on cardiovascular disorders (CVD) in rats with fructose-induced metabolic syndrome (MetS); elucidating how it works.

Materials and Methods: Four groups of male albino rats were allocated into Control, Tocilizumab (8mg/kg/week intraperitoneal), Fructose (10-25% in drinking water), and Fructose +Tocilizumab. After seven weeks of the experiment, measurements of systolic blood pressure (SBP), heart rate (HR), serum fasting glucose, insulin, lipids, IL6 levels, and HOMA test for insulin resistance (IR) were done. Cardiac tissue concentrations of nucleotide-like receptor protein 3 (NLRP3) inflammasome and tissue inhibitor metalloproteinase1 (TIMP1) were estimated. Finally, a histopathological heart examination was performed.

Results: Treatment with tocilizumab significantly alleviated fructose-induced metabolic disorders such as increased HR, glycemic parameters, IR, lipid profile, and IL6. Concurrently, tocilizumab ameliorated cardiac NLRP3 and TIMP1 concentrations; normalizing the histopathological findings of inflammatory infiltration, interstitial fibrosis, and TNF α immunobiological reactivity.

Conclusions: These results show promising cardiovascular protective effects of tocilizumab to be used clinically. However, further cardiovascular investigations such as ventricular contractility, ECG, and vascular reactivity may be required to interpret more benefits of tocilizumab for this purpose.

Keywords: Fructose; IL6; inflammasome; TIMP1; Tocilizumab.

1. Introduction

Metabolic syndrome (MetS) is a multiple risk factor of biochemical, metabolic, physiological, and clinical disorders such as hyperglycemia, reduced insulin sensitivity, abnormal lipid profile, abdominal obesity, hypertension, and inflammation. These disorders can increase the risk of cardiovascular diseases (CVD) by twofold and the risk of diabetes by fivefold [1].

Fructose-induced MetS is strongly correlated with chronic inflammation, characterized by increased levels of blood mononuclear cells and enhanced systemic release of inflammatory cytokines [2]. IL6 is crucial in several metabolic pathways. Increased levels of IL6 in MetS can impact several important conditions such as elevated hepatic glucose secretion, diminished insulin-mediated skeletal muscle glucose uptake, IR, and an increased likelihood of developing hypertension [3].

Tocilizumab, a monoclonal antibody that blocks IL6 receptors (IL6R), is employed to treat inflammatory and autoimmune disorders. Tocilizumab can inhibit both the membrane-bound and soluble versions of IL6R, hence blocking the activity of inflammatory cells and reducing the levels of pro-inflammatory cytokines generated [4].

Inflammasomes are crucial intracellular multiprotein complexes of the innate immune

system, involving a group of receptor proteins known as nucleotide-like receptor proteins (NLRP), involving the NLRP3 inflammasome. The NLRP3 inflammasome plays a significant role in developing and advancing IR, type 2 diabetes mellitus (T2DM), and other metabolic diseases [5].

Matrix metalloproteinases (MMPs) are a class of enzymes that play a role in both normal tissue development and remodeling, as well as in the progression of abnormal conditions. The activity of MMP is regulated by the proteolytic impact of endogenous tissue inhibitors of metalloproteinases (TIMPs) [6]. The expression of TIMP1 is substantially regulated by growth factors and cytokines, such as IL6 and TNF α . Under specific pathological circumstances, there may be an occurrence of improper restructuring of cardiovascular tissue. This can happen either because of excessive production of metalloproteinase or inadequate control of TIMPs. Consequently, a range of disorders such as arthritis and CVD can develop [7].

Because chronic inflammation is observed as a key factor of cardio-metabolic disorders, especially T2DM, MetS, and CVD, treatment of inflammation could represent an important therapeutic target for MetS [8]. This research intends to examine the impact of tocilizumab on cardio-metabolic disorders in male white rats with fructose-induced MetS.

2. Materials and Methods

2.1. Chemicals

Fructose was purchased from El-Farouk Company, El-Fayoum, Egypt. Tocilizumab (Actemra) was purchased from Roche, Basel, Switzerland.

2.2. Methods

Experimental Animals and Design

Twenty-four adult male Wister albino rats weighing 150 ± 20 g was purchased from the National Research Institute, Cairo University, and observed for one week before the study, feeding normal standard diet & drinking tap water ad libitum. The animals were separated into four groups (n=6) as follows: Control and Tocilizumab (8mg/kg intraperitoneal once weekly [9]; rats received normal food and tap water. Fructose group; rats were given 10% fructose in drinking water for the first 39 days, then fructose concentration was modified to 25% in the last 10 days [10,11], and Fructose +Tocilizumab; rats were given fructose and tocilizumab for seven weeks.

Blood Pressure and Heart Rate Measurements

At the end of the seventh week, the SBP & HR of all rats were assessed utilizing the LE 5001 non-invasive blood pressure meter (Panlab; Harvard Apparatus, Barcelona, Spain). The rats were inserted into the restrainer. The tail cuff/transducer was placed into the base of the tail area, the selector switch was switched on and the readings were displayed on the apparatus' screen [12].

Blood Sampling and Histological Study

Under light general anesthesia with diethyl ether, blood samples were collected from retro-orbital veins of fasting rats, centrifuged and serum was separated for determination of biochemical

measurements. The rats were promptly euthanized by cervical dislocation. The heart of each rat was removed and rinsed with an ice-cold saline solution. Subsequently, the heart was divided into two sections. One section was frozen at -60 °C for the quantification of NLRP3 and TIMP1 concentrations, while the other section was fixed in 10% formalin for histopathological analysis. We acquired serial slices that were 4 microns thick and stained them with Hematoxylin and Eosin (H & E) to evaluate histological alterations in the myocardium. Additionally, we utilized Masson's trichrome stain to visualize fibrosis. Then immunohistochemical staining using TNF α antibodies was made. Moreover, the quantitative morphometric study was done by utilizing the "Toup view" image analyzer computer system (China).

Serum glucose, TC, TG, and HDL are measured according to the manufacturer's instructions (Atlas Medical Co., Cambridge, UK). LDL levels were measured by the Friedewald equation [13]: $LDL = TC - (TG / 5) - HDL$. By using ELISA kits, the procedures and methods for measurement of serum insulin (Cat. No. MBS045315), IL 6 (Cat. No. MBS175908), and tissue NLRP3 (Cat. No. MBS 7612469) were according to the manufacturer's instructions of MyBiosource, Southern California, San Diego, USA, while TIMP1 (Cat. No.: E -EL-R0540) was according to Elabscience Biotechnology, Wuhan, China.

Calculation of IR was by homeostatic model assessment for insulin resistance (HOMA-IR) concerning the following equation [14]: $IR = \text{Glucose (mg/dL)} \times \text{Insulin } (\mu\text{U/mL}) / 405$.

2.3. Statistical analysis

Statistical analysis was done using SPSS software Version 22. Data analysis was made utilizing one-way analysis of variance (ANOVA). The comparison among groups was done utilizing the post-

hoc Tukey test. All data were expressed as mean \pm standard deviation (SD) and variances were regarded as significant at P -value <0.05 . Pearson correlation was done to test the correlation among normally distributed variables.

3. Results

In the present study, SBP elevated significantly in the Fructose group as contrasted with both Control and Tocilizumab ($p < 0.05$), while there was no significant variance either among Tocilizumab and Control or between Fructose +Tocilizumab and Fructose groups ($p >0.05$). Regarding HR measurement, the

Fructose group displayed a significant elevation in HR as compared to the Control while Fructose +Tocilizumab significantly decreased it ($p <0.05$). Conversely, there were insignificant changes in HR in the Tocilizumab group as contrasted either to normal or to Fructose Tocilizumab (**Table 1**).

Table 1: Effect of tocilizumab on serum levels of glucose, insulin, IL6, lipids, SBP, and HR levels.

Groups	Control	Tocilizumab	Fructose	Fructose + Tocilizumab
Serum glucose (mmol/ ml)	4.7 \pm .29	4.6 \pm .43 [†]	13 \pm 1.2 [†]	7.8 \pm .68 ^{†‡¶}
Serum insulin (μIU/ml)	7.3 \pm .06	7.3 \pm 1.14 [‡]	18.9 \pm 2.08 [†]	11.1 \pm 1.95 ^{†‡¶}
IL6 (pg/ml)	24.06 \pm 2.37	18.2 \pm 1.94 [‡]	106.1 \pm 9.06 [†]	43.06 \pm 8.29 ^{†‡}
TG (mg/dl)	91.8 \pm 13.66	97.3 \pm 5.68 [‡]	199.2 \pm 9.83 [†]	141.3 \pm 9.09 ^{†‡¶}
TC (mg dl)	139.8 \pm 5.49	131.5 \pm 11.88 [‡]	215.3 \pm 10.23 [†]	173.2 \pm 11.55 ^{†‡¶}
LDL (mg dl)	60.8 \pm 8.36	42.2 \pm 7.24 [‡]	153.2 \pm 10.79 [†]	97.4 \pm 11.43 ^{†‡¶}
HDL (mg dl)	64 \pm 4.05	69.8 \pm 4.12 [‡]	22.3 \pm 4.55 [†]	47.5 \pm 2.88 ^{†‡¶}
SBP (mmHg)	103.8 \pm 8.52	86.7 \pm 15.08 [‡]	256.3 \pm 7.63 [†]	257.3 \pm 33.39 [¶]
HR (beat/ min)	231.7 \pm 16.11	198.5 \pm 39.2 [‡]	240 \pm 21.91 [‡]	240 \pm 21.91 [‡]

N= 6 rats/ group, Data are represented as mean \pm SD, p -value at < 0.05 is considered significant. [†] Significant of all groups contrasted with the control group, [‡] Significant values in comparison with the fructose group, [¶] Significant values in comparison with the tocilizumab group.

Rats who received fructose showed significantly higher values of fasting serum glucose (FSG), fasting serum insulin (FSI), and IR than that of the Control ($p < 0.05$), while there was an insignificant difference between normal control and tocilizumab. Fructose +Tocilizumab decreased significantly all glycemic parameters as compared to the Fructose group, however, the values are still not normalized ($p < 0.05$) (**Table 1 & Figure 1A**).

The present results of the Fructose group revealed a significant increase in TG, TC & LDL levels and a significant decrease in HDL as compared with Control ($p < 0.05$). Results of Tocilizumab and Fructose +Tocilizumab showed significantly decreased TG, and LDL with increased HDL levels as compared with Fructose results, while there were insignificant changes in comparison to Control ($p > 0.05$) (**Table 1**).

Compared with Control, there were significantly increased levels of IL6 and NLRP3 inflammasome in the Fructose group

while TIMP1 concentration significantly decreased ($p < 0.05$), meanwhile, there was an insignificant difference between Tocilizumab and Control results. Fructose +Tocilizumab exhibited a significant decrease in IL6 and NLRP3 alongside increased TIMP1 levels as compared to the Fructose group ($p < 0.05$) (**Figure 1, 1C**).

We further explored the association among IR and serum IL6 as well as cardiac NLRP3 inflammasome of all groups; the results revealed a significant positive correlation of the parameters ($p < 0.05$ and $r > 0.696$), however, there was a significant inverse correlation among IR and cardiac TIMP1 level ($p < 0.05$ and $r < -0.696$) (**Figure 1D-1F**).

Our results showed a significant direct association between IL6 and cardiac NLRP3 concentrations ($p < 0.05$ and $r > 0.696$), while there was a significant negative association among IL6 and TIMP1 levels ($p < 0.05$ & $r < -0.696$) (**Figure 1G, 1H**).

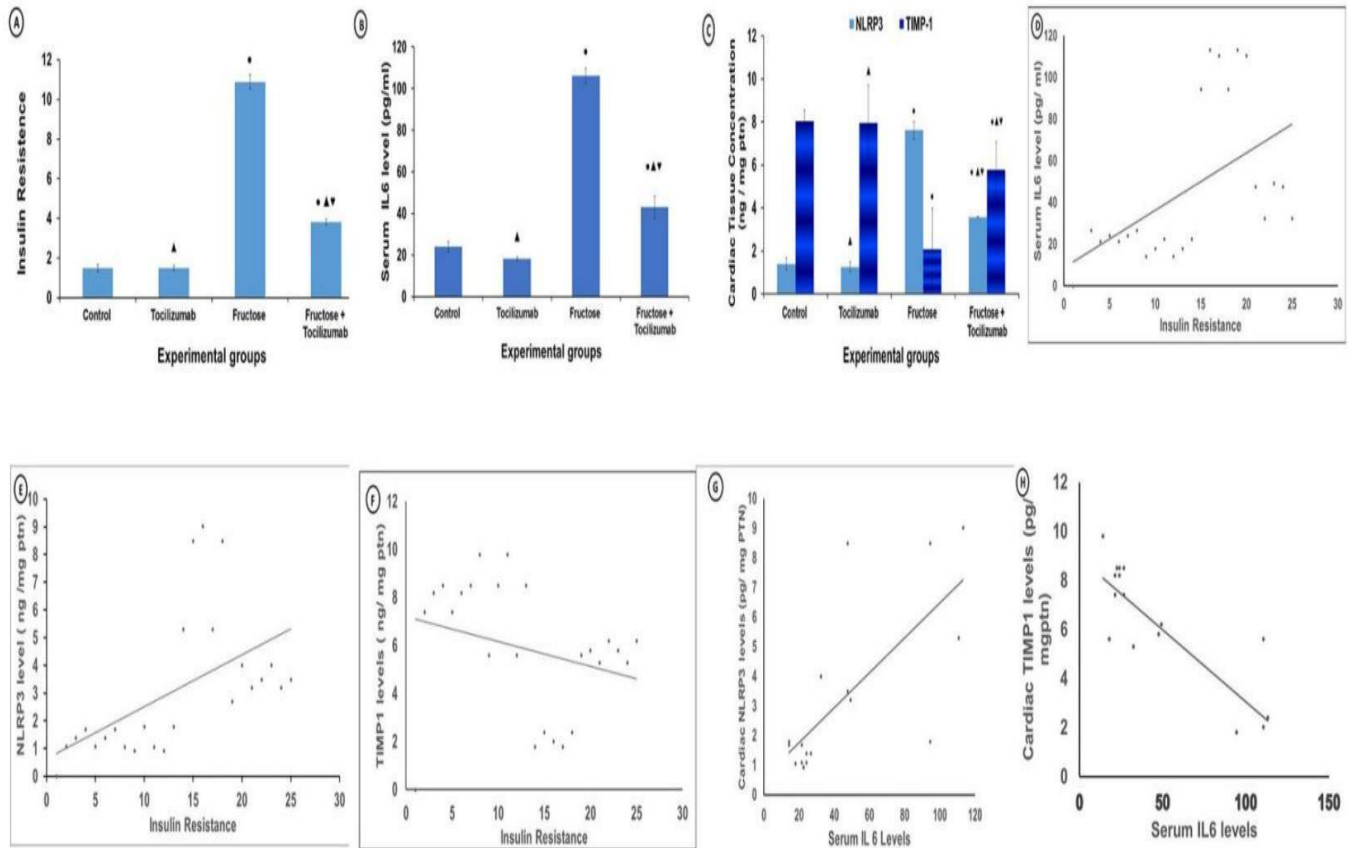


Figure 1: The effect of Tocilizumab on tested variables. (A, B, & C) the effect of tocilizumab on IR, serum IL6 level and cardiac tissue concentration of NLRP3 and TIMP1 respectively; **(D, E, F, G, & H)** The correlation between IR and serum IL6 level, NLRP3 level and TIMP1 level, and between serum IL6 level and cardiac NLRP3 and TIMP1 level respectively.

Histological examination of heart tissues from Control and Tocilizumab rats discovered the normal histological architecture of the myocardial fibers, with centrally located vesicular cigar-shaped nuclei. In contrast, the Fructose group exhibited an irregular wavy

appearance of myocardial fibers, deep acidophilic cytoplasm with darkly stained pyknotic nuclei & inflammatory infiltration with congested blood vessels. Fructose +Tocilizumab rats showed near normal histological appearance (**Figure 2A-2D**).

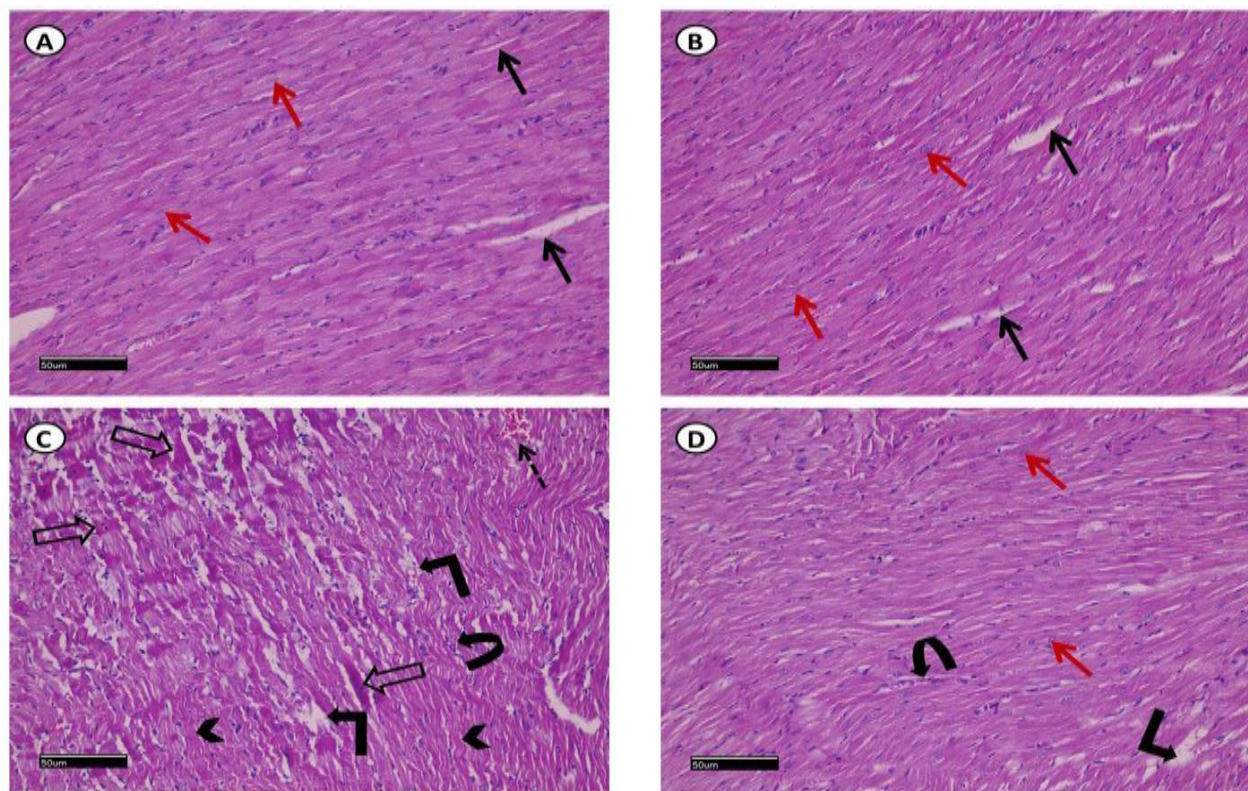


Figure 2: A photomicrograph of H&E stained cardiac muscle sections from all experimental groups Control (A) and Tocilizumab treated group; (B) showing regular arrangement of the myocardial fibers, with centrally located cigar-shaped vesicular nuclei (red arrows). Fructose-treated group; (C) showed a distorted and irregular wavy appearance of myocardial fibers (arrowheads). Wide spaces (right-angled arrows) and inflammatory infiltration (curved arrows) are observed between the myocardial muscle fibers. Congested blood vessels (dashed arrow) could be also detected. in Fructose + Tocilizumab treated group; (D) most of the muscle fibers appeared normal with vesicular nuclei (red arrows). (H&E stain, scale bar = 50µm).

With Masson's trichrome staining the control group displayed minimal collagen fiber deposition between the cardiac muscle fibers and the Tocilizumab group was comparable to the control group with no statistically significant difference in collagen area. The Fructose group (figure exhibited a significant elevation in collagen fiber deposition contrasted with the control group ($p < 0.05$), whereas Fructose +Tocilizumab treatment significantly decreased the collagen deposition in comparison with

Fructose ($p < 0.05$) (**Figure 3A-3D**). With TNF α Immunohistochemical Staining the control group showed nearly negative TNF α immunoreaction. The Tocilizumab group showed scarce cytoplasmic immunoreactivity with insignificant differences compared to control. The Fructose group exhibited a significant increase of TNF α immunostaining ($p < 0.05$) in many myocardial fibers contrasted with the control. Fructose +Tocilizumab decreased the immunoreaction significantly

compared to the fructose group ($p < 0.05$) (Figure 3E-3H).

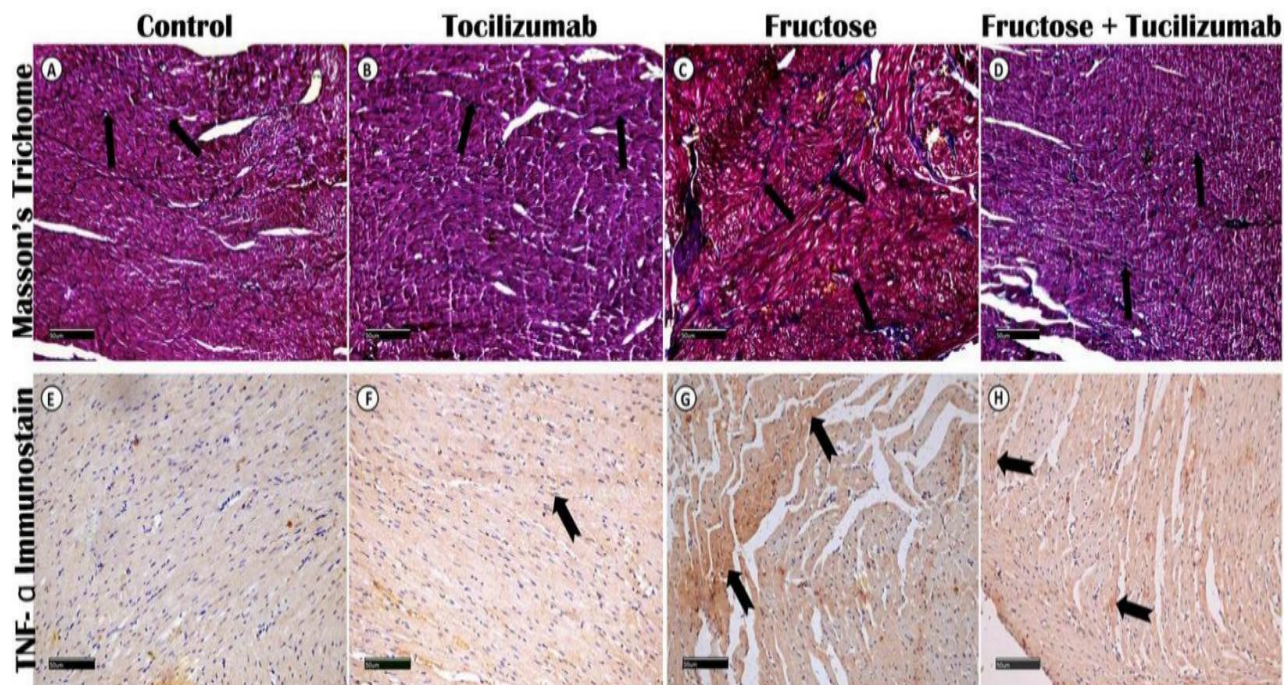


Figure 3: A photomicrograph of cardiac muscle sections from all experimental groups stained with
I) Masson's Trichrome stain: Control (A) and Tocilizumab treated (B) showing mild collagen fibers (thick arrows) between the cardiac muscle fibers. Fructose fructose-treated group (C) exhibited marked collagen fibers deposition (thick arrow). Fructose + Tocilizumab treated (D) showed moderated collagen fibers (thick arrow). **II) TNF- α immunohistochemical staining:** Control (E) showing nearly negative TNF- α immunoreaction. Tocilizumab treated group (F) showing scarce cytoplasmic immunoreactivity for TNF- α (notched arrow). Fructose treated group (G) exhibited TNF- α immunostaining in many myocardial muscle fibers (notched arrows). Fructose + Tocilizumab (H) showed TNF- α immunoreaction in a few myocardial fibers (notched arrow) (Scale bar= 50 μ m).

4. Discussion

In this study, researchers induced a model of MetS by giving subjects fructose in their drinking water for seven weeks. This resulted in significant increases in serum metabolic indices, IL6, SBP, and HR, which is consistent with previous studies [11,12]. The MetS model exhibited decreased cardiac levels of TIMP1, elevated NLRP3 inflammasome

activity, increased TNF α immunoreactivity, and enhanced collagen deposition. Fructose consumption resulted in the emergence of IR and hyperglycemia, potentially caused by the decrease in insulin receptors, leading to a reduction in insulin-dependent glucose utilization and insulin sensitivity [15]. Moreover, the lipogenic impact of fructose,

which causes the accumulation of triglycerides and cholesterol in the liver, might result in glucose intolerance and reduced insulin sensitivity [16].

Research has demonstrated that specific health disorders, including atherosclerosis, cardiovascular events, hypertension, and MetS, are marked by a state of low-level inflammation triggered by IL6 and Tumor TNF α [3,17]. The results suggest that tocilizumab, a medication utilized for inflammatory treatment, effectively ameliorates several cardio-metabolic alterations in fructose-fed rats, except higher SBP, in comparison to rats not administered the drug. The findings indicate that tocilizumab may reduce the levels of IL6, which are elevated by fructose, by inhibiting the signal transduction of soluble IL6R. Consequently, this restraint may result in a decrease in cardiac TNF α and an enhancement in insulin sensitivity in this specific animal model [15].

The observed reduction in cardiac NLRP3 inflammasome levels in rats treated with fructose and tocilizumab is likely attributed to the ability of tocilizumab to inhibit IL6 inflammatory signaling. This aligns with a prior investigation, which showed that inhibiting IL6 resulted in a reduction in the activation of NLRP3 inflammasome in mice afflicted with collagen-induced arthritis [18]. Additional studies have documented that tocilizumab simultaneously inhibits the activation of NLRP3 inflammasome and blocks IL6 receptors [19].

The function of MMPs, which are enzymes that break down proteins inside the extracellular matrix and basement membrane, is regulated by the equilibrium between active MMPs and TIMPs [11]. The results of our study suggest that the intake of fructose is associated with a reduction in myocardial TIMP1 levels and an increase in collagen deposition. This might be attributed to the connection between elevated levels of MMP and heightened concentrations of inflammatory markers, which are associated with various metabolic abnormalities in the MetS model [6]. Our study findings indicate a considerable rise in cardiac TIMP1 levels with the administration of tocilizumab, as compared to the fructose group. Nevertheless, no substantial alterations were detected in comparison to the standard control group. This impact may have been caused by the enhancement of inflammatory markers, such as IL6, TNF α , and NLRP3 inflammasome, which have a role in controlling TIMP1 expression.

Furthermore, our investigation uncovered a notable and favorable correlation between IR and IL6 or NLRP3. This finding aligns with a prior study that demonstrated a direct correlation between the intensity of type 2 diabetes (T2DM) and heightened expression of the NLRP3 inflammasome in adipose tissue. The presence of amyloid polypeptide in pancreatic islets in T2DM may cause the disruption of lysosomes and an increase in oxygen radicals in pancreatic macrophages. This, in turn, activates the NLRP3 inflammasome [20]. According to

Yang et al. (2019), the NLRP3 inflammasome is involved in the advancement of IR and T2DM [5]. The reciprocal relationship between IL6 and NLRP3 inflammasome in immune response and inflammation, along with the notably improved cardiac TNF α immunoreactivity, may elucidate the influence of tocilizumab's anti-inflammatory activities in our MetS model [21].

Following a period of seven weeks of providing a diet high in fructose, the analysis of lipids revealed a notable rise in the levels of TC, TG, and LDL, accompanied by a drop in HDL levels. This agrees with previous findings [15, 22, 23]. Dyslipidemia observed in this study may be caused by hyperglycemia, which alters the intermediary metabolism of lipids and proteins. This typical triad of dyslipidemia, which includes increased TG and LDL with reduced HDL, is commonly associated with IR in patients with MetS [24]. Impaired lipid metabolism primarily raises levels of LDL in the bloodstream, while inflammatory processes aid in the development of atherosclerotic plaques, which can result in serious cardiovascular events. Cholesterol crystals found in atherosclerotic plaques stimulate certain inflammasomes, such as NLRP3, which is known to be highly expressed in the aorta of individuals with atherosclerosis [25]. In addition, in MetS, increased cytokine production

such as IL6 and TNF α may alter TG metabolism, resulting in increased TG and VLDL levels through either excessive synthesis of VLDL or reduced clearance [26].

The IL6 receptor-blocking effect of tocilizumab may stimulate lipid clearance by inducing the VLDL receptor, stimulating lipolysis in hepatic and adipose tissue, and decreasing hepatic lipid synthesis [26]. This, together with modifying NLRP3 inflammasome and TNF α reactivity, could explain the improvement in lipid profile by tocilizumab. Furthermore, the histopathological results in cardiac tissues confirmed the ameliorated state with tocilizumab in fructose-fed rats. These clear improvements were the result of the improvement of all features of MetS.

Conclusion

The results of the present research provide evidence that the biological agent, tocilizumab, can reverse certain changes caused by fructose consumption. These findings confirm the role of IL6 and TNF α in the development of some aspects of the MetS model. Tocilizumab may modulate NLRP3 inflammasome and TIMP1, which are considered strong cardiac inflammatory sensors and potential cardio-protective markers. These promising results indicate that tocilizumab may have cardiovascular protective effects that could be beneficial for clinical use.

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Ethical considerations and consent to participate: The study's experimental protocol was confirmed by the Animal Research Ethics Committee of the Faculty of Medicine, Fayoum

University. Approval No is D 190. The experiment is according to the world guidelines of NAH.

Conflict of interest: There is no conflict of interest to be declared.

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