

RESEARCH

THE EFFECTS OF EXERCISE AND ANTIOXIDANT ON KLOTHO GENE EXPRESSION DURING OXIDATIVE STRESS IN RAT'S CARDIAC TISSUE

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CONFLICTS OF INTEREST

THERE ARE NO CONFLICTS OF INTEREST FOR ANY OF THE AUTHORS.

ABSTRACT:

Obesity has been defined as a state of continuous oxidative stress. There are numerous approaches wherein weight problems immediately affect the cardiovascular device. The klotho gene acts as aging -suppressor gene that protects cells and tissues from oxidative stress. It's now widely known that exercise or antioxidants can prevent oxidative harm by decreasing oxidative stress. This study intended to clarify the effects of exercise and antioxidants on klotho gene mRNA expression in rat's cardiac tissue during oxidative stress .The animals have been divided into five groups. The group I serve as negative control. Group II is a positive control feeding high fat diet (HFD) (Oxidative stress group) .Group III: (HFD) treated with exercise training. Group IV: (HFD) treated with vitamin E as antioxidant and group V: (HFD) treated with exercise training and vitamin E. Serum insulin, glucose, HOMA, total cholesterol and TG were measured. Gene expression of TNF α mRNA expression level in cardiac tissue and Klotho mRNA expression in kidney tissue were performed using Real-time PCR. We demonstrate that combined exercise training with vitamin E treatment for 8 weeks resulted in a significant improvement in the insulin resistance and dyslipidemia. Also, it was associated with significant decrease in TNF α expression in heart and increase in Klotho gene expression in kidney. We concluded that the exercise training and /or antioxidant treatment improved oxidative stress effects in cardiac tissue through up-regulation of mRNA expression of Klotho gene in the kidney and down-regulation of TNF α expression in the heart.

KEYWORDS: Oxidative stress – Klotho gene- Exercise – Cardiac tissue - Gene expression - Real time PCR

1. INTRODUCTION

Oxidative stress may be definitely defined as an imbalance between the systemic manifestations of reactive oxygen species and a biological system's capability to detoxify the reactive intermediators or to repair the resulting harm. In humans, the development of many diseases or exacerbation in their signs and symptoms is thought to be resulting from an oxidative strain. These consist of cancer, atherosclerosis, heart failure; and many others) [1]. Obesity has been defined as a state of chronic oxidative stress. Moreover, oxidative stress has been defined as the link between obesity and related disorders such as insulin resistance and hypertension .During the last decades, the prevalence of obesity has significantly increased reaching epidemic

proportions in many countries [2]. The widespread of cardiovascular disease in obese individuals is indirectly mediated by the increased frequency of certain risk factors like hyperlipidemia, diabetes, and hypertension. However, obesity directly affects the cardiovascular system in several ways) [3]. Obese patients have increased concentration of many adipokines like leptin, resistin and other chemokines like tumor necrosis factor- α , lipoprotein lipase, plasminogen activator inhibitor-1 and interleukin-6. These mediators lead to various adverse effects on the cardiovascular system through the creation of a pro-inflammatory and pro-thrombotic state moreover, it can cause endothelial damage and vascular hypertrophy [4]. Klotho gene is an aging -suppressor gene that prolong lifespan when overexpressed and cause

premature aging-like phenotypes when disrupted in mice, Moreover Klotho protein protects body cells and tissues from oxidative stress, yet the exact mechanism underlying these activities remains to be clarified [5]. Experimental data has shown that physical activity reduces cardiovascular morbidity and mortality and decreases the progression of vascular disease [6], In fact, regular physical results in adaptations inside the antioxidant ability, protecting cells against the unfavourable effects of oxidative stress ; consequently preventing cell damage [7]. An antioxidant is any compound that can defend against the action of an oxidant. Vitamin E is one of the most potent antioxidants [8]. Therefore, the aims of the present study were to clarify the effects of exercise and/or antioxidants on Klotho gene messenger RNA expression during oxidative stress and its effects on cardiac tissue. And to determine the mechanism by which Klotho gene decrease oxidative stress.

2. MATERIALS AND METHODS:

2.1 Experimental animals and protocols:

The prevailing study was carried out on 42 male Albino rats weighing 170-200 gm obtained from the animal residence, Moshtohor Faculty of Veterinary Medicine, Benha University . Strict care and cleansing measures have been applied to maintain the animal in a normal healthy state; the animals have been saved in animal cages beneath the triumphing atmospheric situations and on ordinary balanced diet and tap water until the start of experiment as soon as the experimental protocol became initiated, the rats were divided into five groups (each group contains 7 animals) with group II subdivided into two subgroups (IIa and IIb). Group I (negative control): received regular diet (containing 18.6% protein, 12% lipid, and 69.4% carbohydrates of total caloric requirement) [9] for 16 weeks. Group IIa: received high-fat diet (fat represents 60%, CHO 21.4% and protein 18.6% of total caloric requirements [9] for 16 weeks [10]. Group IIb (positive control): received high-fat diet for 24 weeks without treatment. The remaining three groups of rats were continuously provided with high-fat diet for 24 weeks but undergone treatment only in last 8 weeks. Group III: undergone exercise training. Group IV: received vitamin E. Group V: The rats undergone exercise training and received vitamin E. Food consumption of all rats was measured daily. Body weights were recorded weekly. The following acclimatization to swimming in a water tank and according to swimming protocol of Reynolds et al., [11] rats in group III and group V were exercised at 15 min swim interval, the training time were slowly increased to 45 min day⁻¹ over 1 week period, 5 days week⁻¹ for a total 8 weeks. In group IV and V Vitamin E was

dissolved in olive oil and given daily orally at a dose of 1 gm/kg [12].

2.2 Samples preparation:

After an overnight fasting, the animals were anesthetized with diethyl ether. The animals were fixed on operating table and the blood sample, heart and kidney biopsies were taken.

Serum preparation: after the blood sample (2ml were taken from the right ventricle, samples were allowed to clot at room temperature and serum was separated by using centrifugation at 3000 rpm for 15 min and stored at - 20oc in darkish packing containers for biochemical analysis of serum glucose, insulin, TG, and cholesterol.

The kidney and heart biopsies: Biopsies of kidney and the heart were taken and without delay located in Cryo tubes and stored in RNA later solution (Qiagen, GmbH, Hilden, Germany) at 10 µL per 1 mg of tissue at -80°C for further processing.

2.3 HOMA (homeostatic model assessment):

The approximating equation for insulin resistance used a fasting blood sample and was derived by use of the insulin-glucose product, divided by a constant: $\text{Glucose (mg/dl)} / 18 \times \text{Insulin} (\mu\text{IU/ ml}) / 22.5$ [13].

2.4 Assessment klotho and tumor necrosis factor TNF α genes expressions:

2.4.1 Total RNA extraction:-

Total RNA extraction was done by using total RNA Purification Kit from Jena Bioscience GmbH, according to the manufacturer instructions with about 30 mg tissue collected in a microcentrifuge tube and then 300 µl of lysis buffer containing 2ME (2 Mercapto Ethanol) which was homogenized using rotor Tissue Raptor (Qiagen, GmbH).

Spectrophotometric Quantification of RNA: Measure the absorbance using Nanodrop spectrophotometer Thermo Scientific (U.S.A) at A260 and A280. The concentration of RNA sample was measured 44ug/ml A260 (Wilfinger et al., 1997)[14]. The ratio of the reading at (A260/ A280) provides an estimate of the purity of RNA. Pure RNA has an A260/A280 ratio of 1.9-1.3.

2.4.2 Two steps RT-PCR:

1st step: Template RNA (5ul) and distilled water (15 ul) put into Maxine RT Premix tube (Intron Biotechnology). cDNA synthesis (Reverse transcription) reaction by G-storm thermal cycler (England) was finished at a temperature of 45°C for 60 min followed by RTase inactivation step at 95°C for 5 min . The product was diluted by adding thirty ml nuclease-free water. 2nd step: RT-PCR was accomplished by the usage of ABI 7900HT fast real-time PCR (Applied Biosystem USA), the

prepared reaction components were achieved in 96 well PCR plate (micro Amp @ 90 well optical reaction plate with Barcode, code 128). Singleplex reaction was done using qPCR Green Master from (Jena Bioscience GmbH), using real-time cycler conditions of 95°C for five min. (Initial denaturation), followed by thirty-five cycles of 95°C, 30 S, 55°C, 1 min and 72°C, with 30 S for denaturation, annealing and extension steps respectively. The primer sequences were from (5'-3') for all genes, Klotho gene forward 5' -CGT GAA TGA GGC TCT GAA AGC - 3' reverse 5'- GAG CGG TCA CTA AGC GAA TAC G -3', TNF forward 5'-CTG TAG CCC ACG TCG TAG C -3' reverse 5'- TTG AGA AGG TCC ATG CCG TTG - 3'and GAPDH as endogenous control forward 5'- CGA CTT CAA CAG CAA CTC CCA CTC TTC C -3' reverse- TGG GTG GTC GAG CAG GGT TTC TTA CTC CTT - 3' [15].

Data analysis: According to the RQ manager program 1.2 ABI SDS software (ABI 7900HT), the records are produced as sigmoid shaped amplification plots in which the variety of cycle is plotted in opposition to fluorescence (while the use of linear scale). Because the samples of manage organization are used as calibrators, the expression levels are set to at least one. But due to the fact the gene expression stages were plotted as log₁₀ values (log₁₀ of one is zero), the expression degree of the calibrator samples appear as 0 inside the graph. Due

to the fact that relative portions of the klotho or TNFα gene are normalized in opposition to the relative quantities of the endogenous control GAPDH gene fold expression changes have calculated by the usage of the equation $2^{-\Delta\Delta CT}$ (Livak et al., 2001)[16].

2.5 Statistical analysis:

The collected data was tabulated and statistically analyzed. The results are presented as means ± Standard Error (SE) (ANOVA test). All analysis was performed using the Statistics Package for Social Sciences (SPSS) and Microsoft office Excel is used for data processing and data analysis. After we calculate the “t” value we consulted the “t” distribution table to get the “p” (probability value). Statistical significance was accepted at P value <0.05 or lower.

3. RESULTS:

Our study showed that Consumption of HFD for 16 weeks resulted in a significant increase (p <0.05) in bodyweight of HFD group (group IIa) compared to the control group associated with development of insulin resistance manifested by significant increase (p <0.05) in serum glucose levels, serum insulin level (p<0.001) and HOMA-IR (p <0.05) and dyslipidemia manifested by significant (p <0.05) increase in serum total cholesterol and triglycerides as showed in (table 1).

Table (1): Comparison of the effect of consumption of high-fat diet (group IIa) and negative control group (group I) .

Variable	Mean ± SD; (range)		t	p*
	negative control group I (n=7)	High-fat diet IIa (n=7)		
Weight at the end of study(gm)	200 ± 7.65; (190-210)	323 ±24.30; (300-360)	3.14	<0.01*
Serum glucose (mg/dl)	102.71 ± 3.82; (99-110)	196.86 ±4.45; (190-202)	3.14	<0.01*
Serum insulin (μIU / ml)	4.66 ± 0.97; (3.8-6.2)	6.79 ± 1.73; (4.9-9.2)	2.24	<0.05*
HOMA	1.17 ± 0.24; (1-1.5)	3.31 ±0.85; (2.4-4.5)	3.17	<0.01*
Serum Total Cholesterol(mg/dl)	39.14 ± 1.95; (37-42)	47.71 ± 6.05; (41-54)	2.7	<0.01*
Serum Triglycerides(mg/dl)	63.86 ± 8.8; (55-78)	83.71 ± 26.9; (62-142)	2.11	<0.05*
TNF log ₁₀ RU	4.61 ± 0.01; (4.6-4.63)	4.77 ± 0.01; (4.76-4.78)	3.17	<0.01*
Klotho log ₁₀ RU	4.57 ± 0.01; (4.56-4.59)	3.94 ± 0.05; (3.89-4)	3.16	<0.01*

t: Student's Test. R.U: Relative Unit

Our study showed that exercise training for 45minutes /day for 8weeks resulted in a significant (p<0.05) decrease in the weight of the exercise group (group III) compared to the positive control group (group IIb), the decrease in weight was associated with an improvement in the insulin resistance state as shown by by significant (p<0.05) decrease in serum glucose, serum insulin and HOMA -IR as showed in (table 2 and figure 2)

Table (2): Comparison among the last four groups (high-fat diet IIb, exercise III, antioxidant IV, and group V).

Variable	Mean \pm SD (range)				P between groups
	High-fat diet IIb (n=7)	Exercise III (n=7)	Antioxidant IV (n=7)	Exercise and antioxidant V (n=7)	
Weight at the end of the study (gm)	355.57 \pm 34.26 (319-400)	216.29 \pm 3.45 (211-220)	355.57 \pm 34.26 (319-400)	206.14 \pm 2.12 (204-210)	p <0.05* for all
Serum glucose (mg/dl)	210.57 \pm 8.24 (199-220)	107.86 \pm 4.49 (102-114)	118.14 \pm 2.12 (115-120)	98 \pm 3.70 (90-101)	p <0.05* for all
Serum insulin (μ IU / ml)	6.25 \pm 1.52; (4.5-8.2)	4.28 \pm 1.32; (2-5.5)	6.07 \pm 1.28; (4.5-8.1)	4.01 \pm 0.70; (3.1-5.3)	p1,3,4,6,<0.05* P2,5 >0.05
HOMA	3.16 \pm 0.71; (2.3-4.3)	1.17 \pm 0.37; (0.5-1.6)	1.75 \pm 0.42; (1.1-2.3)	1.03 \pm 0.16; (0.9-1.3)	p <0.05* for all except p 5>0.05
Serum Total Cholesterol(mg/dl)	50.71 \pm 3.9 (48-58)	37.42 \pm 5.02 (28-43)	40 \pm 7.4 (28-50)	40.14 \pm 3.8 (35-47)	p1: p3 <0.05 p4:p6 >0.05
Serum Triglycerides (mg/dl)	108.3 \pm 30.7 (70-140)	42.57 \pm 10.96; (31-60)	54.4 \pm 15.5 (40-76)	53.7 \pm 13.27 (43-71)	p1: p3 <0.05* p4:p6 >0.05
TNF α log ₁₀ RU	4.79 \pm 0.01 (4.78-4.80)	4.48 \pm 0.01 (4.46-4.49)	4.78 \pm 0.01 (4.77-4.79)	4.69 \pm 0.01; (4.68-4.7)	p <0.05* for all except p 2 >0.05
Klotho log ₁₀ RU	3.92 \pm 0.05 (3.87-3.98)	4.32 \pm 0.05 (4.27-4.38)	4.68 \pm 0.05 (4.63-4.74)	5 \pm 0.05 (4.95-5.06)	p <0.05 for all

p1: between (group IIb) and (group III). **p2:** between (group IIb) and (group IV).

p3:between (group IIb) and (group V). **p4:**(group III) and (group IV).**p5:**between (groupIII) and (group V). **p6:** between (group IV) and (group V).

Table (3): Comparisons between the effect of exercise training for 8 weeks (group III) and the effect of combined exercise training and treatment with vitamin E for 8 weeks (group V) in rats received HFD for 24 weeks.

Variable	Mean \pm SD; (range)		t	p*
	Exercise III (n=7)	Antioxidant IV (n=7)		
Weight at the end of the study (gm)	216.29 \pm 3.45; (211-220)	355.57 \pm 34.26; (319-400)	3.13	<0.01
Serum glucose (mg/dl)	107.86 \pm 4.49; (102-114)	118.14 \pm 2.12; (115-120)	3.14	<0.01
Serum insulin (μIU / ml)	4.28 \pm 1.32; (2-5.5)	6.1 \pm 1.52; (3.9-8.2)	1.98	<0.05
HOMA	1.17 \pm 0.37; (0.5-1.6)	1.75 \pm 0.42; (1.1-2.3)	2.18	<0.05
Serum Total Cholesterol	37.42 \pm 5.02; (28-43)	40 \pm 7.4; (28-50)	0.905	>0.05
Serum Triglycerides	42.57 \pm 10.96; (31-60)	54.4 \pm 15.5; (40-76)	1.35	>0.05
TNF log₁₀ RU	4.48 \pm 0.01; (4.46-4.49)	4.78 \pm 0.01; (4.77-4.79)	3.17	<0.01
Klotho log₁₀ RU	4.32 \pm 0.05; (4.27-4.38)	4.68 \pm 0.05; (4.63-4.74)	3.14	<0.01

There was an increase in TNF α expression level in cardiac tissue by 1.5 folds and there was a decrease in Klotho gene expression in renal tissue by 0.263 fold in group IIa HFD when compared to control group (group I). Figure (1)

There were increases in Klotho expression levels by 2.4, 5.52, 11.67 folds and decreases in TNF expression by 0.51, 0.97, 0.76 folds in groups III, IV, V respectively when compared to group IIb HFD (positive control) Figure (2)

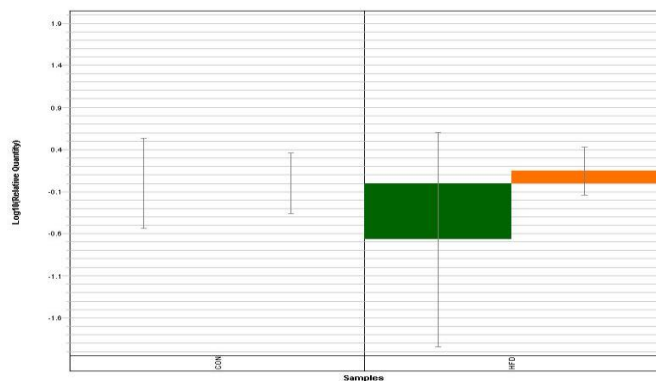


Figure 1: Gene expression levels of Klotho mRNA & TNF α for high-fat diet samples. Because control samples are used as calibrators, the expression stages are set to at least one. However due to the fact the expression stages were blotted as log -10 values (and the log 10 of 1 is zero), the expression stage of the control sample seems as zero in the graph. Due to the fact the relative portions of the Klotho mRNA & TNF α mRNA are normalized in opposition to the relative portions of the GAPDH (endogenous manipulate), the expression level of the endogenous manipulate is zero; there aren't any bars for GAPDH.

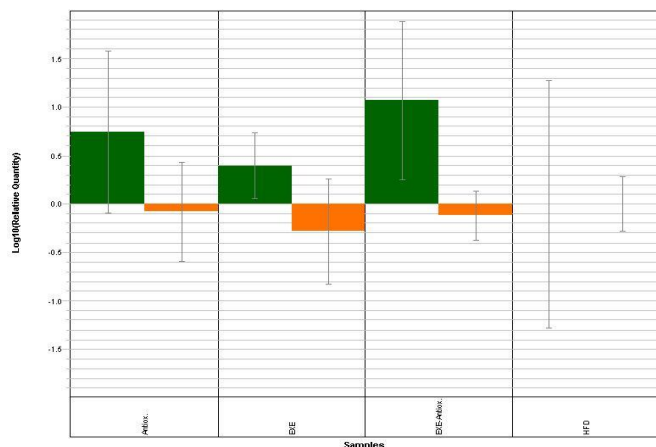


Figure2: Gene expression levels of Klotho mRNA & TNF α for different groups. Because HFD samples are used as calibrators, the expression stages are set to one. But because the expression Stages were blotted as

log -10 values (and the log 10 of 1 is 0), the expression level of the HFD samples appear as zero in the graph.

On comparing the effect of exercise training for 8 weeks (group III) to the effect of vitamin E treatment for 8 weeks (group IV) on rats received HFD for 24 weeks, it is clear that there are significant decreases in body weight, serum glucose level, serum insulin level and HOMA-IR as described in table 3. There are no significant differences in serum total cholesterol and serum triglycerides. The TNF expression level was significantly decreased ($P < 0.01$) but the Klotho mRNA expression is significantly increased ($P < 0.01$) in the exercise group (group III) when compared with the antioxidant group (group IV).

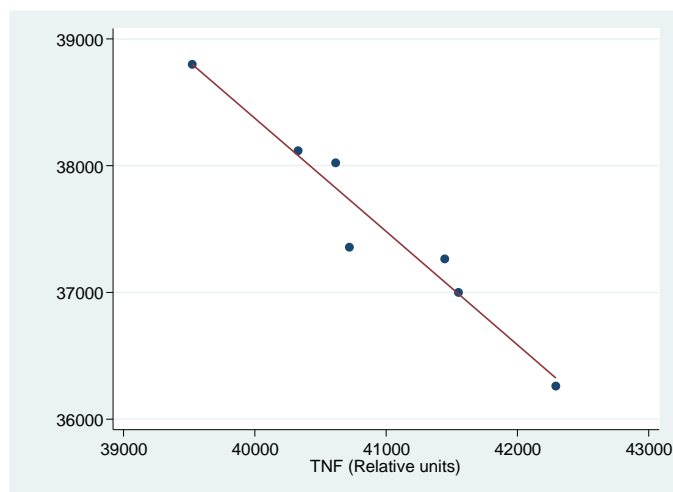


Figure 3: Correlation coefficient between Klotho gene expression and TNF α by relative units in HFD group.

There were significant ($P > 0.01$) negative correlations between Klotho gene and TNF α mRNAs expression in each group figure (3). Otherwise, there was no significant correlation between Klotho gene expression and any other variable among all groups.

4. DISCUSSION

Developing proof suggests that exercising prevents oxidative damage via reducing oxidative stress, a critical aspect in inflammation and hypertension. What remains uncertain is exactly how exercise modulates redox fame and how it affects proinflammatory processes [17]. We hypothesized that the expression of Klotho gene may be play an important interlocking mediator in reducing the oxidative stress produced by HFD intake

The significant findings after consumption of HFD for 16 weeks are in agreement with Guo et al., [18] who reported that supplying an HFD to rats for 16 weeks produces a dyslipidemic profile, elevates serum fasting glucose, insulin and TNF α concentrations, and results in

significant body weight gain and hepatic steatosis. [19], also reported that mice on HFD developed greater body weight relative to regular chow diet-fed mice at 16 and 32 weeks, respectively manifesting impaired glucose tolerance, insulin resistance, and cardiac ceramide accumulation by 16 weeks. One of the feasible mechanisms for obesity-induced insulin resistance is that obesity is a proinflammatory condition in which hypertrophied adipocytes and adipose tissue-resident immune cells (basically lymphocytes and macrophages) both contribute to elevated circulating stages of proinflammatory cytokines. The overweight related state of persistent low-grade systemic inflammation termed “metabolic inflammation,” is considered a key point inside the pathogenesis of insulin resistance in human beings and rodent animal models [20]. Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines such as adiponectin, transforming growth factor beta (TGF β), interleukin (IL)-10, IL-4, IL-13 and IL-1 receptor antagonist (IL-1Ra). In dissimilarity, obese adipose tissue primarily releases proinflammatory cytokines like TNF- α , IL-6, leptin, visfatin, resistin, angiotensin II and plasminogen activator inhibitor 1 [21]. The proinflammatory adipokines modulate insulin resistance either directly by upsetting the insulin signaling pathway or indirectly via stimulation of inflammatory pathways [22]. Also, utilization of HFD for 16 weeks resulted in significant increase in the expression level of TNF- α by 1.5 folds in cardiac tissue of the group IIa compared to the control group I as showed in (table 1 and figure 1). These are in agreement with Noyan-Ashraf et al., [19] who reported that the hearts of mice received HFD for 16 weeks manifested increased expression of the inflammatory cytokine TNF- α . Kawasaki et al., [23] also reported that the expression levels of TNF- α in adipose tissue were dramatically increased in mice received HFD for 16 weeks. One of the possible mechanisms for The increased expression level of TNF - α is that triglycerides and/or free fatty acids may be inducers of TNF- α expression because feeding rats high-fat diet results in a significant increase in TNF- α mRNA and protein in fat pads [24]. Another explanation may be the recruitment of macrophages into adipose tissue [25]. The huge accumulation of adipose tissue macrophages (ATMs), representing as much as 40% of the cells in overweight adipose tissue, determines domestically extended levels of pro-inflammatory cytokines, which includes TNF- α and IL-6, which sustain insulin resistance in a paracrine manner [26]. Obesity is associated with a low-grade chronic inflammation characterized by infiltration of monocytes / macrophages in skeletal, cardiac and adipose tissues [19]. So we may postulate that the increased expression of TNF in cardiac tissue with HFD may be due to its infiltration with

macrophages. The 2nd step was to study the effect of HFD on Klotho gene expression. Azuma et al., [27] reported that the aging-suppressor gene klotho is predominantly expressed in the kidney no matter species. In our work, consumption of HFD for sixteen weeks resulted in significant decrease in klotho gene expression within the kidney of HFD group (group IIa) in comparison to the controls as confirmed in (table 1 and figure 1). These findings are confirmed by Sastre et al., [28] who reported that a significant reduction in Klotho mRNA and protein expression was observed in kidneys from hypercholesterolemic ApoE KO (apolipoprotein E knockout) mice fed an HC (high cholesterol) diet as compared with controls. The possible mechanism for the reduction in Klotho mRNA in HFD group may be explained as ox-LDL decrease Klotho expression in tubular cells through activation of ERK (Extracellular Signal-Regulated Kinases) and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathways [29]. Another possible mechanism was reported by Delfin et al., [30] who demonstrated that the enhanced inflammation in adipose tissue in obesity, exemplified by increased TNF- α level, influences the actions of FGF21 through strong down-regulation of β -Klotho.

Increasing the duration of consumption of HFD to 24 weeks resulted in no change in different parameters except for significant increase in serum glucose level and TNF- α expression level in cardiac tissue (table 2 and figure 2). These results are in agreement with Alhusseini et al., [31] who reported that increasing the duration of consumption of HFD for 20, 22 and 24 weeks resulted in no significant increase in the body weight or insulin resistant state on comparing them with the consumption of HFD for 16 weeks. The 3rd step was to study the effect of exercise training on HFD induced oxidative stress. The significant improvement of insulin resistance in exercise group is highly suggesting that the exercise training is one of the main strategies to reduce insulin resistance. Our results are in agreement with Gray et al., [32], Alhusseini et al., [31] who reported that exercise resulted in an improvement in insulin resistance in rats. Also Hawley and Lessard [33] who reported an improvement in insulin resistance with exercise in humans. There are numerous postulated mechanisms through which exercise might also improve insulin resistance. Physical training reduced serum TNF- α concentrations, TG, TC, and FFA. The increased TNF- α inhibits the insulin receptor signal transduction and insulin-stimulated glucose transportation in adipose tissue [34] [35]. Also, TNF- α initiates and aggravates IR via TNF receptor (TNFR) [36]. Blocking TNF- α effect not only increased insulin sensitivity and promoted insulin receptor tyrosine kinas activity but also reduced the plasma FFA level In Zucker rats [37]. Our study

showed that exercise training for 8 weeks also improved dyslipidemia manifested by significant ($p < 0.005$) decrease in serum total cholesterol and TG as showed in (table 2). This is in agreement with Ghasemof et al., [38] who reported that aerobic exercises resulted in a significant decrease in triglyceride levels, cholesterol, LDL and a significant increase in HDL (High-density lipoprotein). The improvement in dyslipidemia with exercise training was explained by Wang and Nakayama [39] who reported that exercising causes the rapid activation of the sympathetic nervous system, the result of which is released epinephrine and norepinephrine hormones that lead to lipolysis ultimately. Exercising also leads to a reduction in serum insulin concentrations with regard to lipolysis inhibiting function of the insulin hormone. On the other hand, exercising stimulates the growth hormone, which is another important factor for lipolysis process. Meissner et al., [40] also demonstrated that Voluntary wheel running increased cholesterol turnover in healthy mice owing to an increased fecal bile acid excretion and decreased intestinal cholesterol absorption. The exercise training also resulted in significant decrease ($p < 0.001$) in the expression level of TNF mRNA in cardiac tissue as showed in (table 2 and figure 2). Our results are in agreement with several experimental studies which reported that regular exercise decreases TNF mRNA expression [35], [41]. Down-regulation of the TNF- α by exercise training could be explained by the following mechanism. Interleukine-6 (IL-6) is the first cytokine to be released into the circulation during exercise, and its levels increase steadily in response to exercise [42]. IL-6 exerts anti-inflammatory effects through its inhibitory effects on TNF- α and IL-1 β , and activation of interleukin-1 receptor antagonist (IL-1ra) and IL-10 [43]. IL-10, in turn, reduces the production of several proinflammatory cytokines, such as TNF- α and IL-1 β [44]. On the other hand, our study showed that exercise training for 8 weeks resulted in significant increase ($p < 0.001$) in Klotho gene expression in the kidney as showed in (table 2 and figure 2). These are in agreement with Matsubara et al., [45] who reported that aerobic exercise training increases plasma Klotho levels in postmenopausal women. The underlying mechanism for upregulation of klotho gene expression by exercise is unexplained yet. However, in our study the exercise training downregulates TNF- α mRNA expression. Inflammatory cytokines such as TNF-like weak inducer of apoptosis (TWEAK) and TNF down regulate Klotho expression through an NF κ B dependent mechanism [46]. So it's possible that exercise training upregulates Klotho gene expression by downregulation of TNF- α mRNA expression. As regard to study the effect of vitamin E treatment on HFD induced oxidative stress. As our study showed that treatment with vitamin E for 8 weeks didn't result in

significant change in the body weight of the antioxidant group (group IV) compared to the positive control group (group IIb) as showed in (table 2). This is in agreement with Kedziora-Kornatowska et al., [47] as they revealed that administration of vitamins C and E did not affect body weight of diabetic rats. However, in the same group, the insulin resistance was significantly improved manifested by significant decrease ($p < 0.05$) in serum glucose levels, HOMA-IR ($p < 0.05$) and decrease in serum insulin level (although statistically nonsignificant) as showed in (table 2), and these results are in agreement with Manning et al [48] as they reported that vitamin E improved insulin sensitivity and the associated features of insulin resistance in overweight individuals and they explained that the mechanisms of the improvement in insulin resistance were by decreasing cellular oxidative stress, altering membrane properties, and decreasing inflammatory activity. Treatment with vitamin E also results in significant ($P > 0.05$) decrease in serum total cholesterol and TG (table 2) and these results confirm that the antioxidant vitamins (vitamin A, vitamin C and vitamin E) cause decrease in serum total cholesterol, low-density lipoprotein-cholesterol (LDL-C) and triglycerides concentrations [49]. The inhibition of hypercholesterolemia may have resulted from the increased excretion of fecal bile acids in rats treated with antioxidant (Resveratrol) [50]. On the other hand treatment with the antioxidant vitamin E didn't affect the TNF- α expression level in cardiac tissue (table 2). On the other hand, our study showed that treatment with the vitamin E resulted in significant ($p < 0.05$) increase in Klotho mRNA expression by 5.5 folds in the kidney (figure 2). This is in agreement with Hu et al., [51] as they reported that oral administration of antioxidant (troglitazone: antidiabetic with antioxidant properties) for 10 weeks significantly upregulates renal Klotho mRNA expression in OLETF rats. Cheng et al., [52] also reported that the reduced expression of klotho in cultured renal tubular cells of Streptozotocin-induced diabetic rats was reversed by antioxidant (Tiron). Hu et al. 2012 [51] proposed that overexpression of PPAR- γ (Peroxisome proliferator-activated receptor gamma) can be the underlying mechanism of increased renal Klotho expression by Antioxidant.

The last step was to study the effect of combined exercise training with vitamin E treatment for 8 weeks on HFD induced oxidative stress. Our study showed that combined exercise training with vitamin E treatment resulted in a significant ($p < 0.05$) decrease in body weight together with improvement in parameters of insulin resistance. Henriksen. [53] reported that The interaction between exercise training and the antioxidant (R-ALA) on the glucose transport system likely results from an additive effect of the two interventions on IRS-1 (Insulin receptor substrate 1) biosynthesis and

functionality, leading to the greatest effect of insulin to stimulate PI3-kinase (phosphatidylinositide 3-kinase). Combined exercise training with vitamin E treatment for 8 weeks also resulted in significant decrease in serum total cholesterol and TG. Also, this combined regime resulted in significant decrease in TNF-alpha mRNA expression level in cardiac tissue and the significant increase in Klotho mRNA expression in the kidney (table 2 and figure2). These results are in correlation with our discussed previous results. On the other hand by comparing the different parameters in the exercise group(group III) with that of the antioxidant group(group IV) our study showed that the body weight, insulin resistance parameters and TNF expression in cardiac tissue were significantly lower in the exercise group (group III) compared to the antioxidant group (group IV),while Klotho mRNA expression was significantly higher in the antioxidant group (group IV) compared to the exercise group(group III) and no significant difference was detected between the two groups regarding serum total cholesterol and TG as showed in (table 2). The difference between the results of the two groups may be explained by the possibility that the mechanisms by which the exercise training affects the body weight, insulin resistance and TNF expression level may be more efficient than that of antioxidant treatment while the reverse may be right regarding the effect of antioxidant treatment on Klotho expression [54]. These findings support the assumption that increased Klotho gene expression plays a role in reducing oxidative stress. And we assume that this effect may be mediated even partially through down-regulation of TNF gene expression.

5. CONCLUSION

We conclude that the exercise training and /or antioxidant treatment improves HFD induced oxidative stress in cardiac tissue and up-regulates mRNA expression of Klotho gene in the kidney and this suggests that the effect of the exercise training and antioxidant treatment on oxidative stress may be mediated even partially through increased Klotho mRNA gene expression. We assume that this role of Klotho may be mediated through down regulation of TNF gene expression.

6. RECOMMENDATIONS

Further studies are needed on the effect of exercise training on Klotho gene expression to find out other mechanisms by which the exercise up-regulate Klotho gene expression.

Further studies are needed on the effect of Klotho gene on oxidative stress to find out other mechanisms by which Klotho reduces oxidative stress.

Further investigations are needed to find out the effect of exercise training with different durations and/or intensities on Klotho gene expression and whether this improvement would be reversed after stoppage of exercise.

Further investigations are needed to find out the effect of different types of antioxidants for different durations on Klotho gene expression.

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