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Effects of omega-3 fatty acids and pioglitazone combination on insulin resistance through fibroblast growth factor 21 in type 2 diabetes mellitus

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ABSTRACT

Fibroblast growth factor 21 (FGF21) is an effective regulator of glucose and lipid metabolism. It is mainly regulated by peroxisome proliferator activated receptors, and is widely associated with cases of insulin resistance as obesity and type 2 diabetes (T2D). Our study aimed to investigate the potential effects of omega-3 fatty acids, pioglitazone, and their combination on serum and liver FGF21 concentrations, and its hepatic gene expression in a rat model of T2D. We also studied the modulating effects of these treatments on blood glucose, lipid profile, and insulin resistance. T2D was induced in male Sprague–Dawley rats by combination of high fat diet and low dose streptozotocin (35 mg/kg). Diabetic rats were treated with omega-3 fatty acids (10%W/W diet), pioglitazone (20 mg/kg), and their combination for a period of 4 weeks. Serum FGF21 concentration was significantly increased in diabetic rats. In contrast, hepatic FGF21 concentration, and gene expression were significantly decreased. Omega-3 fatty acids, pioglitazone, and their combination significantly decreased serum FGF21. Omega-3 fatty acids and combination therapy significantly decreased liver FGF21 concentration, with non-significant changes in gene expression. On the other hand, pioglitazone significantly increased hepatic FGF21 concentration and gene expression. Omega-3 fatty acids, pioglitazone and their combination significantly improved lipid profile. Pioglitazone and combination significantly decreased blood glucose levels and improved insulin resistance. In conclusion, this study introduces the first evidence regarding the antidiabetic effects of omega-3 fatty acids and pioglitazone combination, such effects are mediated through FGF21.

1. Introduction

Diabetes prevalence is increasing at an accelerating rate approaching epidemic levels. Currently, there are 382 million diabetic patients worldwide. In the Middle East and North Africa, 1 in 10 adults have diabetes. Egypt is one of the top 10 countries in the number of people with diabetes (7.5 million in 2013) and the number is expected to increase to 13.1 million by 2035 [1]. Diabetes is characterized by hyperglycemia resulting

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from disorders in insulin secretion, insulin action or both [2]. Type 2 diabetes (T2D) is a heterogeneous, progressive disorder initially characterized by glucose intolerance and compensatory hyperinsulinemia, which in later stages progresses to insulin resistance and impaired beta cell function [3].

Type 2 Diabetes, which accounts for 90–95% of diabetic cases, is largely caused by social and lifestyle factors that can be readily controlled [1,2]. Obesity leads to an increase in the adipose tissue mass. This in turn triggers insulin resistance in fat, skeletal muscle, and liver leading to T2D [4]. A medical approach is not always sufficient for T2D management and lifestyle modification should be considered.

Omega-3 fatty acids (ω-3 fatty acids) have shown to possess anti-inflammatory, and lipid lowering effects, suggesting a possible beneficial effect in management of T2D and its complications [5,6]. Several animal studies have shown that administration of the ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), prevented the development of insulin resistance, obesity, and dyslipidemia [6–8]. These effects are mediated via interaction with peroxisome proliferator-activated receptor α (PPAR-α) [9]. PPAR is a nuclear receptor that plays important roles in adipocyte differentiation, lipid and carbohydrate metabolism through transcriptional regulation of different genes [10]. Other studies in T2D patients and rat models of insulin resistance showed that ω-3 fatty acids failed to reverse insulin resistance [6,11–14].

Pharmacological management of T2D aims to increase insulin secretion or improve insulin sensitivity [15]. Pioglitazone belongs to a class of drugs named thiazolidinediones, which improve insulin sensitivity, lipid metabolism, and glucose homeostasis through activation of PPAR-γ [16].

Fibroblast growth factor 21 (FGF21) is a recently identified hormone that has an important role in glucose and lipid homeostasis [17–19]. Rat FGF21 is a circulating protein derived from a 208-amino acid mature protein encoded by the FGF21 gene located in chromosome 1 [20]. FGF 21 is detected in plasma, so it is proposed to be secreted into circulation acting as a true hormone. Its activity depends on binding to FGF receptors (FGFR) and a cofactor called β-Klotho, a transmembrane protein whose expression is stimulated during development of preadipocytes to adipocytes [21]. The cofactor β-Klotho is predominantly expressed in metabolic organs including liver, white adipose tissue, and pancreas [22]. FGF21 has glucose-lowering effects through several mechanisms. It stimulates glucose uptake in differentiated adipocytes via the induction of glucose transporter-1 (GLUT1) [17,23,24]. Glucose uptake induced by FGF21 is additive and independent of insulin. It has also been reported that FGF21 might act on glucagon metabolism, leading to decreased hepatic glucose production, and increased liver glycogen [25]. Finally, FGF21 was shown to preserve β-cell function and survival [26].

Fibroblast growth factor 21 is produced by the liver and white adipose tissue, under the influence of PPAR-α and PPAR-γ respectively [27,28]. It was reported that serum FGF21 concentration is significantly increased in case of hypertriglyceridemia, insulin resistance, and metabolic syndrome, indicating a state of FGF21 resistance [29].

Peroxisome Proliferator Activated Receptor-γ agonists have been shown to lower blood glucose, total cholesterol, and triglycerides concentrations and improve insulin resistance. Whether these effects are mediated via modulation of hepatic FGF21 is still unclear. The aim of this study is to investigate the potential anti-diabetic effects of combining a PPAR-α agonist (ω-3 fatty acids) and a PPAR-γ agonist (pioglitazone), and whether these effects are mediated through modulation of FGF21.

2. Materials and methods

2.1. Drugs and chemicals

Omega-3 fish oil (containing EPA 180 mg and DHA 120 mg) was purchased from Lifeplan (Lutterworth, Leicestershire, England). Pioglitazone was supplied in the form of pharmaceutical product (Diabetin tablets, Uni Pharma co., Cairo, Egypt). Streptozotocin (STZ) was purchased from Sigma–Aldrich (St. Louis, Mo., USA). The feeding ingredients were obtained from commercial sources and were of analytical grades.

2.2. Animals

Fifty male Sprague–Dawley (SD) rats weighing 150–190 g were allowed free access to food and tap water. Rats were kept under standard conditions of temperature (22 ± 2°C) and relative humidity (55 ± 5%) with 12-light/12-dark cycles. The animal care and experiments described in this study were complied with " Research Ethics Committee" Faculty of Pharmacy, Mansoura University, Egypt, in accordance with " Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985).

2.3. Induction of T2D

Experimental rats were maintained on a high fat diet (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) ad libitum. The high-fat diet (HFD) was prepared and composed as described by Srinivasan et al. (2005) [30]. After 1 month on high fat diet, experimental rats were injected intraperitoneally with freshly prepared STZ (35 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5) after an overnight fasting [30]. Control rats were injected with citrate buffer only. Rats were continued on high fat diet until the end of study. To overcome the hypoglycemia during the first 24 h after STZ injection, diabetic rats were given 5% glucose solution instead of drinking water. Only animals with persistent blood glucose levels higher than 250 mg/dL for 7 days after STZ administration were considered diabetic.

2.4. Experimental design

Animals were divided into:

1. Control group: maintained on normal pellet diet (3.15 kcal/g), and intraperitoneally received single dose of citrate buffer (0.1 M, pH 4.5).
2. Diabetic rats were divided into four groups as follows:
   (i) Diabetes: in which diabetes was induced as previously mentioned.

(ii) Diabetes + ω-3: diabetic rats received ω-3 fatty acids (EPA and DHA) (10% of dietary intake of rats) [31].
(iii) Diabetes + pio: diabetic rats treated with pioglitazone (20 mg/kg body weight) [32].
(iv) Diabetes + ω-3 + pio: diabetic rats treated with a combination of both ω-3 fatty acids (10% of dietary intake of rats) and pioglitazone (20 mg/kg body weight).

Treatments were administered by orogastric gavage, and continued for 4 weeks.

2.5. Body weight

Body weights were monitored weekly during the study.

2.6. Collection of blood and tissue samples

At the end of the experiment, blood samples were withdrawn from thiopental-anesthetized animals via retro-orbital puncture and centrifuged at 1200 × g for 10 min at 4 °C for serum preparation. Immediately after sacrificing the rats, dissection was done for isolation of the liver. Sections of liver tissues were collected, immediately immersed in liquid nitrogen, and stored at −80 °C for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. 0.5 g of liver tissues were homogenized in 5 ml ice-cold phosphate buffer saline (0.02 M, pH 7.4) (10% w/v), centrifuged at 1500 × g for 15 min at 4 °C and frozen at −80 °C until further analysis of FGF21 by ELISA technique.

2.7. Analysis of serum total cholesterol, triglycerides, and blood glucose

Serum triglycerides and total cholesterol concentrations were measured using commercial kits (Spinreact, Spain). Blood glucose was measured from tail vein using (ACCU-CHECK GO, Roche Diagnostics, Mannheim, Germany) glucometer.

2.8. Measurement of fasting insulin concentration (mU/L)

Serum insulin concentration was measured by ELISA using commercially available kit (MyBioSource, Inc., California, USA).

2.9. Calculation of homeostasis model assessment of insulin resistance (HOMA-IR) HOMA-IR was calculated using the following formula

HOMA-IR = Fasting plasma glucose (mg/dl) × fasting insulin (mU/L)/405.

HOMA-IR, first described by Matthews et al. (1985), is a method for estimating insulin sensitivity [33].

2.10. Measurement of FGF21 concentration (pg/ml) using ELISA

FGF21 concentration was measured in serum and liver homogenate by ELISA using a commercially available kit (MyBioSource, Inc., California, USA).

2.11. Gene expression of FGF21

- RNA isolation and c-DNA synthesis

Total RNA was isolated from liver tissues using easy-RED total RNA extraction kit (iNtRON Biotechnology, *Seongnam*, Korea) according to the manufacturer's protocol. RNA was extracted with chloroform, followed by centrifugation at 4 °C to separate into aqueous and organic phases. RNA pellets were recovered from the aqueous phase by isopropyl alcohol precipitation and resuspended in nuclease-free water (Thermo Fisher Scientific Inc.). A total of 1 μg of RNA was used for c-DNA synthesis using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. The resulting c-DNA products were stored at − 20 °C.

- Real-time RT-PCR

Real-time RT-PCR was performed using Piko Real-PCR System (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. For a final reaction volume of 20 μL, the following reagents were added: 4 μL HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, Tartu, Estonia), 1 μL each of forward and reverse 10 μM primers, 12 μL nuclease free water and 2 μL cDNA template. The primers used were designed using PREMIER Biosoft, USA and the specific gene sequences were obtained from Pubmed (Entrez Gene). Primer sequence is described in Table 1.

The amplification was performed as follows: 1 cycle of initial activation of DNA polymerase at 95 °C for 15 min, then repeated three-step cycling for 40 cycles: denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and elongation at 72 °C for 20 s.

mRNA levels were quantified by using comparative C_T method ($2^{-\Delta\Delta C}{}_T$ method). The data were normalized to GAPDH RNA to account for differences in reverse transcriptase efficiencies and the amount of template in the reaction mixtures. The amplified samples were subjected to electrophoresis through 2% agarose gel.

Table 1 – Primers sequence and direction.

Gene	Direction	Sequence	Reference sequence
FGF21	Forward	5′-ACAGATGACGACCAGGACAC-3′	NC_005100.4
	Reverse	5′-TAGAGGCTTTGACACCCAGG-3′	
GAPDH	Forward	5′-CCATCAACGACCCCTTCATT-3′	NC_005103.4
	Reverse	5′-CACGACATACTCAGCACCAGC-3′	

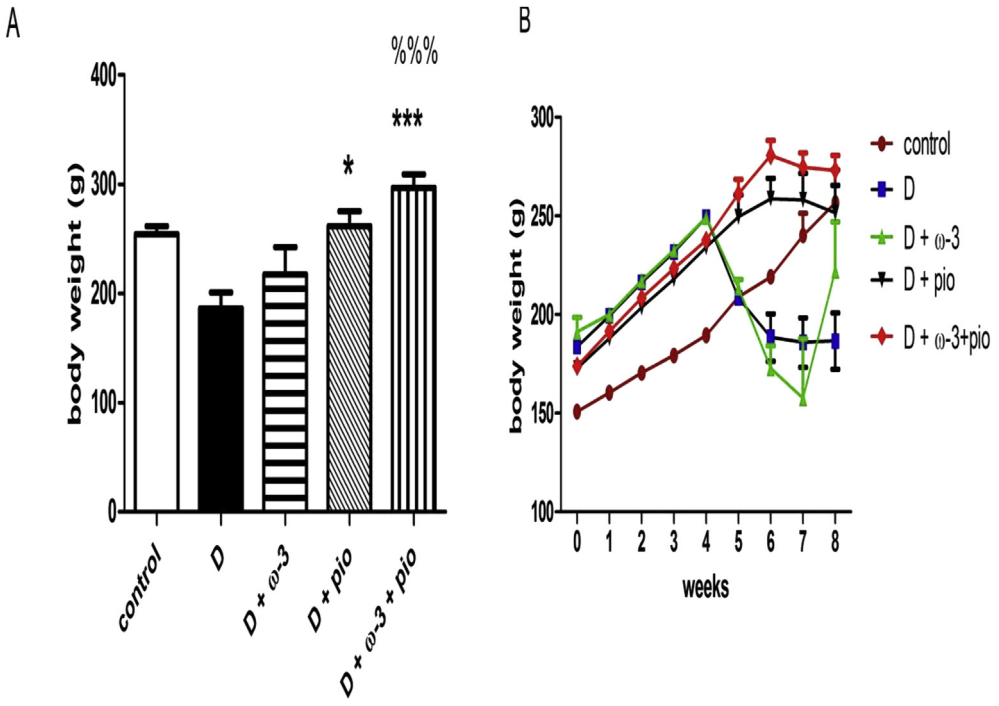

Fig. 1 – Effects of ω-3 fatty acids (10% of diet), pioglitazone (20 mg/kg body weight) and their combination on body weight of diabetic (D) animals. (A) A bar chart showing body weight at the end of the study. (B) A curve showing the weights of animals in each group monitored weekly. Data are represented as means ± SEM of 6–8 animals/group. Statistically significant differences are indicated as: $^{*}p < 0.05$, $^{***}p < 0.001$, compared to D group. %%% $p < 0.001$, compared to D + ω-3 group. D:diabetes. D + ω-3: diabetes + ω-3 fatty acids. D + pio: diabetes + pioglitazone. D + ω-3 + pio: diabetes + ω-3 fatty acids + pioglitazone.

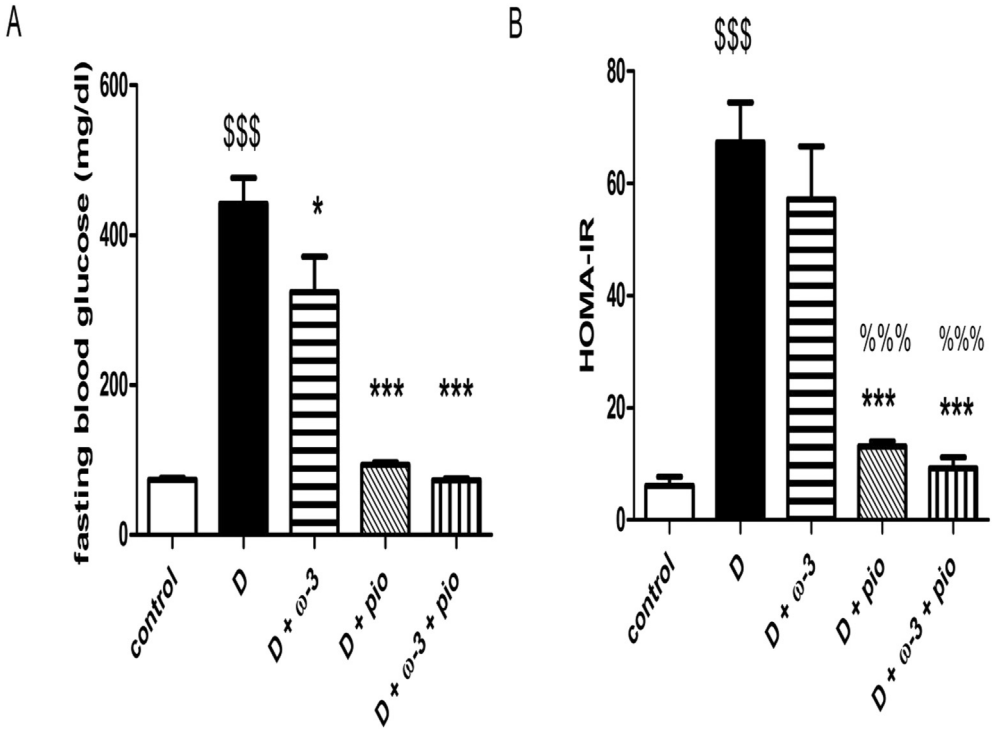

Fig. 2 – Effects of ω-3 fatty acids (10% of diet), pioglitazone (20 mg/kg body weight), and their combination on fasting blood glucose (A) and HOMA-IR (B) of D animals. Data are represented as means ± SEM of 6–8 animals/group. Statistically significant differences are indicated as: $$$ $p < 0.001$, compared to control group. $^{*}p < 0.05$, $^{***}p < 0.001$, compared to D group. %%% $p < 0.001$, compared to D + ω-3 group. D: diabetes D + ω-3: diabetes + ω-3 fatty acids. D + pio: diabetes + pioglitazone D + ω-3 + pio: diabetes + ω-3 fatty acids + pioglitazone.

3. Statistical analysis

Data are expressed as means ± standard error of mean (SEM) in each group. Statistical evaluations of the results, were carried out by means of one way analysis of variance, followed by Bonferroni multiple comparison test. The correlational analysis was performed using Pearson Correlation. Statistical tests were performed using Statistical Package for the Social Sciences (SPSS) version 13 (Chicago, IL, USA). Statistical significance was taken at $P < 0.05$. Graphing was carried out using Graphpad Prism software (Graphpad Software Inc., San Diego, USA).

4. Results

4.1. *Effects of ω-3 fatty acids, pioglitazone, and their combination on body weight*

Diabetic (D) group showed an increase in body weight followed by acute weight loss after STZ injection (Fig. 1a). At the end of study, pioglitazone monotherapy significantly increased the body weight compared to diabetic (D) group ($p < 0.05$). ω-3 fatty acids treated group (D+ω-3) showed non-significant change from diabetic (D) group. Combination group (D+ω-3+pio) showed highly significant increase in the body weight compared to diabetic (D) group ($p < 0.001$) (Fig. 1b).

4.2. *Effects of ω-3 fatty acids, pioglitazone, and their combination on fasting blood glucose concentration*

ω-3 fatty acids monotherapy significantly reduced fasting blood glucose concentrations, compared to diabetic (D) rats ($p < 0.05$), but remained higher than normal fasting blood glucose concentration. Both pioglitazone and combination therapy caused highly significant decrease in fasting blood glucose concentration, compared to diabetic (D) group ($p < 0.001$) (Fig. 2a).

4.3. *Effects of ω-3 fatty acids, pioglitazone, and their combination on HOMA-IR*

Diabetic (D) rats exhibited highly significant increase in HOMA-IR compared to control group ($p < 0.001$), indicating a state of insulin resistance. ω-3 fatty acids monotherapy

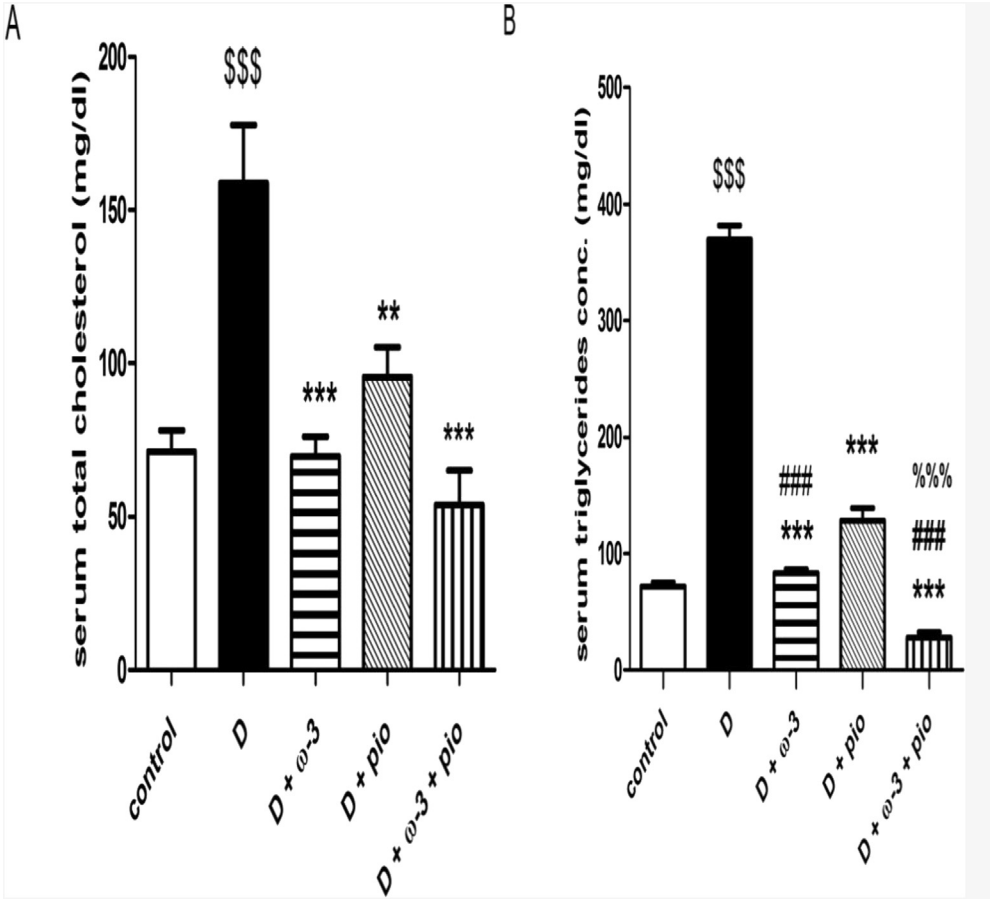

Fig. 3 – Effects of ω-3 fatty acids (10% of diet), pioglitazone (20 mg/kg body weight) and their combination on serum total cholesterol (A) and serum triglycerides (B) of D animals. Data are represented as means ± SEM of 6–8 animals/group. Statistically significant differences are indicated as: $$$ $p < 0.001$, compared to control group. ** $p < 0.01$, *** $p < 0.001$, compared to D group. ### $p < 0.001$, compared to D + pio. %%% $p < 0.001$, compared to D + ω-3 group. D: diabetes. D + ω-3: diabetes + ω-3 fatty acids. D + pio: diabetes + pioglitazone. D + ω-3 + pio: diabetes + ω-3 fatty acids + pioglitazone.

showed non-significant change compared to diabetic (D) group, suggesting absence of insulin-sensitizing effects. Pioglitazone and combination therapy showed a highly significant decrease in HOMA-IR ($p < 0.001$) (Fig. 2b).

4.4. *Effects of ω-3 fatty acids, pioglitazone, and their combination on serum total cholesterol (mg/dl) and serum triglycerides (mg/dl)*

Diabetic (D) rats showed a highly significant elevation in serum total cholesterol and triglycerides concentrations, compared to control group ($p < 0.001$). ω-3 fatty acids, pioglitazone, and combination therapy caused a highly significant reduction in serum total cholesterol and triglycerides, compared to diabetic (D) group ($p < 0.001$). Interestingly, combination therapy showed a highly significant decrease in triglycerides concentration than either ω-3 fatty acids or pioglitazone alone ($p < 0.001$) (Fig. 3a and b).

4.5. *Effects of ω-3 fatty acids, pioglitazone, and their combination on serum FGF21 concentration (pg/ml) and hepatic FGF21 concentration (pg/g liver tissue)*

Serum FGF21 was increased ≈ 2-fold in diabetic (D) group, compared to control group. ω-3 fatty acids, pioglitazone, and combination therapy significantly lowered serum FGF21, compared to diabetic (D) group ($p < 0.001$) (Fig. 4a). As for hepatic FGF21 concentration, diabetic (D) rats showed a highly significant decrease, compared to control group ($p < 0.01$). Pioglitazone monotherapy caused a highly significant increase in hepatic FGF21 concentration ($p < 0.001$), while ω-3 fatty acids therapy resulted in a highly significant decrease whether administered alone or in combination with pioglitazone ($p < 0.001$) (Fig. 4b).

4.6. *Effects of ω −3 fatty acids, pioglitazone, and their combination on hepatic FGF21 mRNA expression*

Diabetic (D) group showed highly significant decrease in FGF21 mRNA expression in liver ($p < 0.001$). Pioglitazone treated group showed highly significant increase in hepatic FGF21 mRNA expression ($p < 0.001$), while both ω-3 and combination therapy showed non-significant changes from diabetic (D) group (Fig. 5).

4.7. *Correlation studies*

Data in Table 2 show the correlational analysis of glucose, HOMA-IR index, serum total cholesterol, serum triglycerides, serum FGF21 concentration, hepatic FGF21 concentration and FGF21 relative gene expression.

As shown in Fig. 6, serum FGF 21 showed significant positive correlation with blood glucose ($r = 0.5$, $p < 0.01$), HOMA-IR ($r = 0.390$, $p < 0.05$).

As shown in Table 2, serum FGF 21 showed significant positive correlation with serum total cholesterol ($r = 0.695$,

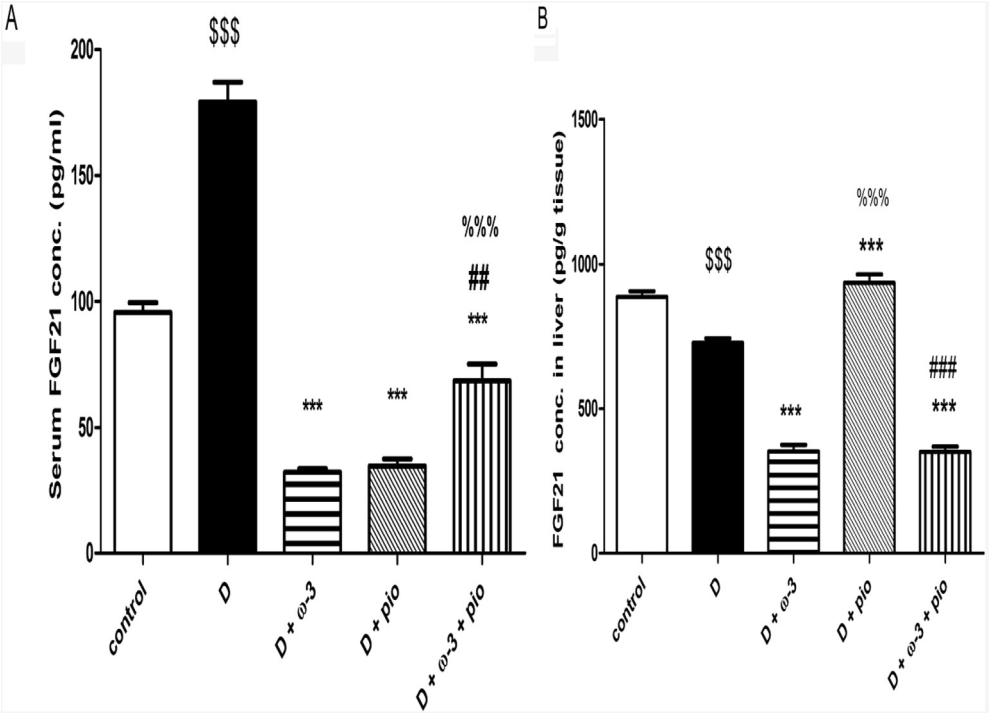

Fig. 4 − Effects of ω-3 fatty acids (10% of diet), pioglitazone (20 mg/kg body weight), and their combination on serum FGF21 concentration (pg/ml) (A), hepatic FGF21 concentration (pg/g tissue) (B). Data are represented as means ± SEM of 6–8 animals/group. Statistically significant differences are indicated as: $$$ $p < 0.001$, compared to control group. *** $p < 0.001$, compared to D group. ## $p < 0.01$, ### $p < 0.001$, compared to D + pio. %%% $p < 0.001$, compared to D + ω-3 group. D: diabetes. D + ω-3: diabetes + ω-3 fatty acids. D + pio: diabetes + pioglitazone. D + ω-3 + pio: diabetes + ω-3 fatty acids + pioglitazone.

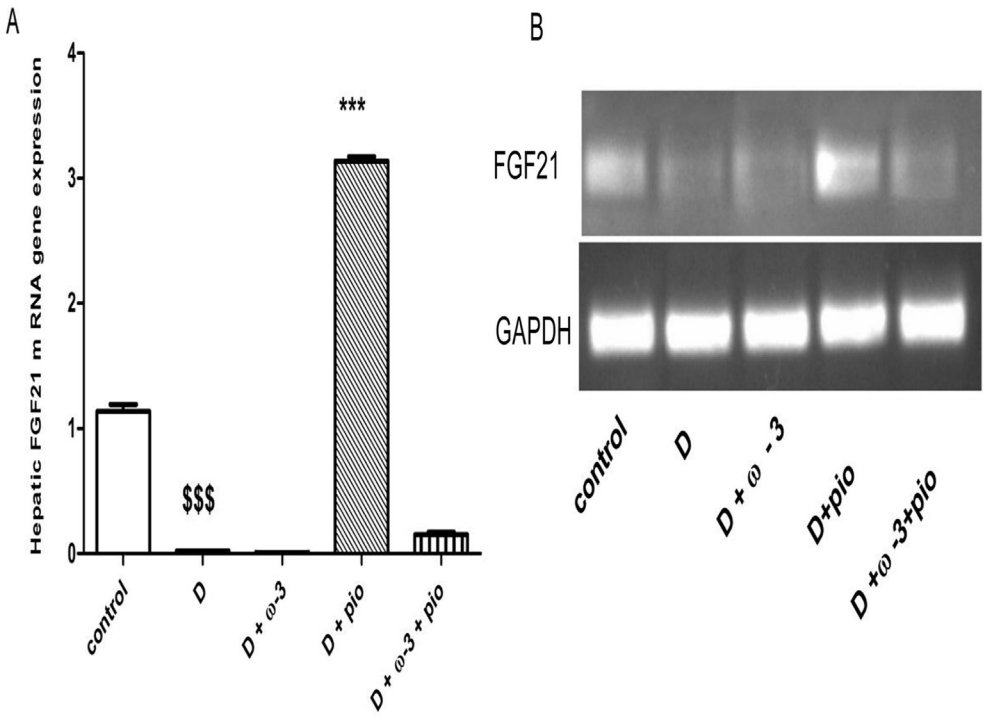

Fig. 5 – Effects of ω-3 fatty acids (10% of diet), pioglitazone (20 mg/kg body weight), and their combination on FGF21 mRNA expression (A), gel electrophoresis (B). Data are represented as means ± SEM of 4 animals from each group. Statistically significant differences are indicated as: $$$ $p < 0.001$, compared to control group. *** $p < 0.001$, compared to D group. D: diabetes. D + ω-3: diabetes + ω-3 fatty acids. D + pio: diabetes + pioglitazone. D + ω-3 + pio: diabetes + ω-3 fatty acids + pioglitazone.

$p < 0.01$) and serum triglycerides ($r = 0.775$, $p < 0.01$). A significant positive correlation was found between hepatic FGF21 concentration and its mRNA expression ($r = 0.448$, $p < 0.05$). As for HOMA-IR, it is positively correlated with serum total cholesterol ($r = 0.55$, $p < 0.01$), serum triglycerides ($r = 0.648$, $p < 0.01$) and blood glucose ($r = 0.963$, $p < 0.01$).

5. Discussion

Combining life style changes with pharmacological therapy has become mandatory for management of diseases associated with metabolic disorders. Previous studies showed the effectiveness of combining ω-3 fatty acids with thiazolidinediones, pioglitazone and rosiglitazone, in mice fed a high-fat diet, possibly through induction of adiponectin [34].

In this study, we generated a rat model for T2D using a high-fat diet followed by low dose STZ as previously described by Srinivasan et al. (2005) [30]. T2D was confirmed by hyperglycemia, mild hyperinsulinemia, and a significant increase in HOMA-IR index in diabetic rats. Our study is the first to investigate the potential anti-diabetic effects of ω-3 fatty acids and pioglitazone combination through modulating FGF21. We have also correlated FGF21 changes with different metabolic parameters.

Table 2 – Correlational analysis of the studied parameters.

	Blood glucose (mg/dl)	HOMA-IR index	Serum total cholesterol (mg/dl)	Serum triglycerides (mg/dl)	Serum FGF21 (pg/ml)	FGF21 conc. in liver (pg/gm tissue)	Hepatic FGF21 m-RNA expression
Blood glucose (mg/dl) (r)	1	0.963**	0.567**	0.724**	0.500**	−0.203	−0.435
HOMA-IR index (r)	0.963**	1	0.550**	0.648**	0.390*	−0.252	−0.345
Serum total cholesterol (mg/dl) (r)	0.567**	0.550**	1	0.851**	0.695**	0.378*	−0.163
Serum triglycerides (mg/dl)(r)	0.724**	0.648**	0.851**	1	0.775**	0.304	−0.222
Serum FGF21 (pg/ml) (r)	0.500**	0.390*	0.695**	0.775**	1	0.219	−0.460*
Liver FGF21 (pg/g.tissue) (r)	−0.203	−0.252	0.378*	0.304	0.219	1	0.448*

*$p < 0.05$.
**$p < 0.01$.

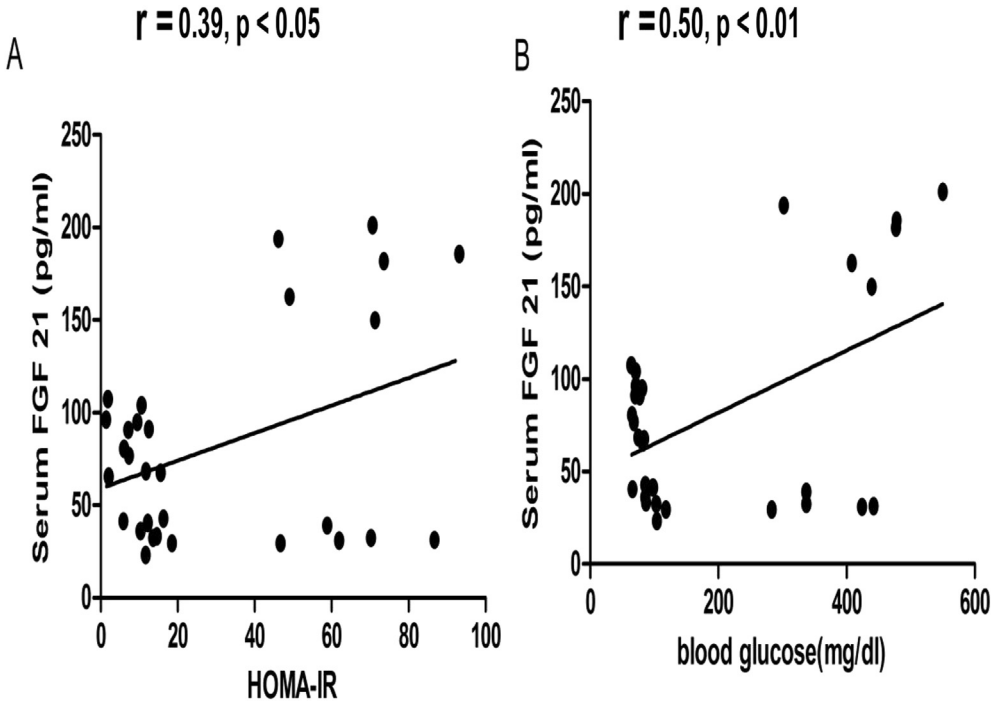

Fig. 6 – Correlation between serum FGF21 (pg/ml) and HOMA-IR (A) and blood glucose (B).

We have shown that ω-3 fatty acids and pioglitazone combination exerted an additive improvement in insulin sensitivity. This is in agreement with previous studies that employed pioglitazone and rosiglitazone in combination with ω-3 fatty acids in rats fed a high-fat diet [34,35]. However, in our study, rats were treated with much lower doses of pioglitazone in the combination therapy and managed to revert insulin resistance.

The effect of ω-3 fatty acids on insulin resistance has been studied extensively leading to contradicting results. Consistent with our results, human studies suggested that none of ω-3 fatty acids improve insulin sensitivity [36,37]. Similarly, Gillam, et al. (2009) [11] found no beneficial effects of ω-3 fatty acids on insulin sensitivity in fa/fa Zucker rats, a rat model of insulin resistance. In a rat model of T2D induced by high fat diet, low dose STZ, Coppey et al. (2012) found that partial replacement of saturated fats with menhaden oil, a natural source of ω-3 fatty acids failed to improve impaired glucose utilization [38].

On the other hand, several murine models of insulin resistance have shown beneficial effects of ω-3 fatty acids in prevention or reversal of insulin resistance. In an obesity model of insulin resistance and fatty liver disease, dietary intake of fish oil (8% wt/wt) had insulin sensitizing actions in adipose tissue and liver [39]. In high sucrose diet-fed rats, 7% fish oil supplementation reversed the insulin resistance [40]. However, the aforementioned studies are models of insulin resistance or pre-diabetes state, none of them represent a model of T2D, as we had in this study.

Pioglitazone caused significant reduction in blood glucose concentrations as shown in various animal species by acting as insulin sensitizer [41]. ω-3 fatty acids significantly decreased fasting blood glucose, but still in levels higher than normal ones. In a rat model of T2D, treatment with menhaden oil did not significantly change blood glucose levels, compared to untreated diabetic rats [38].

Combining ω-3 fatty acids with pioglitazone resulted in a strong synergistic triglyceride-lowering effect. This is in accordance with a previous study that showed an additive effect with higher pioglitazone doses [34]. In the current study, ω-3 fatty acids significantly lowered total cholesterol and triglycerides, compared to the untreated group. ω-3 fatty acids exert potential hypocholesterolemic effect through inhibition of key enzymes related to cholesterol synthesis and transfer such as 3-Hydroxy-3-methylglutaryl reductase and acyl-CoA:cholesterol acyltransferase [42]. ω-3 fatty acids selectively lower triglycerides by increasing glucose flux to glycogen, increasing mitochondrial β-oxidation, and decreasing triglycerides synthesis, an effect that is mediated partially by PPAR-α activation [43]. Pioglitazone was found to significantly lower total cholesterol and triglycerides concentrations, compared to diabetic rats. PPAR-γ activation controls a variety of genes involved in different pathways of lipid metabolism such as fatty acid uptake, fatty acid oxidation, lipolysis, and lipoprotein assembly and transport [44–46].

Pioglitazone and combination therapy significantly increased body weight, compared to diabetic group. Weight gain is a major side effect of pioglitazone treatment, since PPAR- γ increases feeding by reducing leptin levels, a hormone that regulates satiety center [43].

Several studies have reported the use of FGF21 as an interesting external therapeutic agent for modulating insulin

resistance. Evidence from animal-based studies suggested that FGF21 possesses beneficial effects on carbohydrate and lipid metabolism, improving obesity and diabetes [17,47]. As well, administration of recombinant FGF21 has been shown to reverse hyperglycemia, hyperinsulinemia, and dyslipidemia in *ob/ob* and diet-induced obese mice and in diabetic monkeys [48–50].

Consistent with our results, many studies showed that circulating FGF21 concentrations are elevated in insulin-resistant states, such as glucose intolerance and T2D [51–54], indicating a possible compensatory increase of FGF21 to overcome insulin resistance. PPAR is a pivotal regulator of hepatic FGF21 [27,55]. A recent study showed that FGF21 could be regulated by PPAR-γ activation in pancreatic islets of mice [56].

We investigated the possibility of regulating FGF21 concentrations in serum and liver and its gene expression by combining ω-3 fatty acids as a PPAR-α agonist with pioglitazone as a PPAR-γ agonist. Our results have shown that pioglitazone, ω-3 fatty acids, and their combination significantly decreased serum FGF21, compared to diabetic group, alleviating the state of FGF21 resistance. Consistent with our results, Villarroya et al. (2014) showed that long-term dietary treatment with ω-3 fatty acids significantly decreased FGF21 protein levels in plasma, compared to high fat diet group [57]. A study on patients with T2D showed that addition of pioglitazone to exenatide therapy significantly reduced plasma FGF21 levels [58].

Fibroblast growth factor 21 was previously thought to be a hepatic hormone, preferentially expressed in the liver. However, it was reported that other tissues as adipose and muscle tissue are important sources of FGF21 production [19,59–61]. In our study, serum FGF21 concentration was increased in

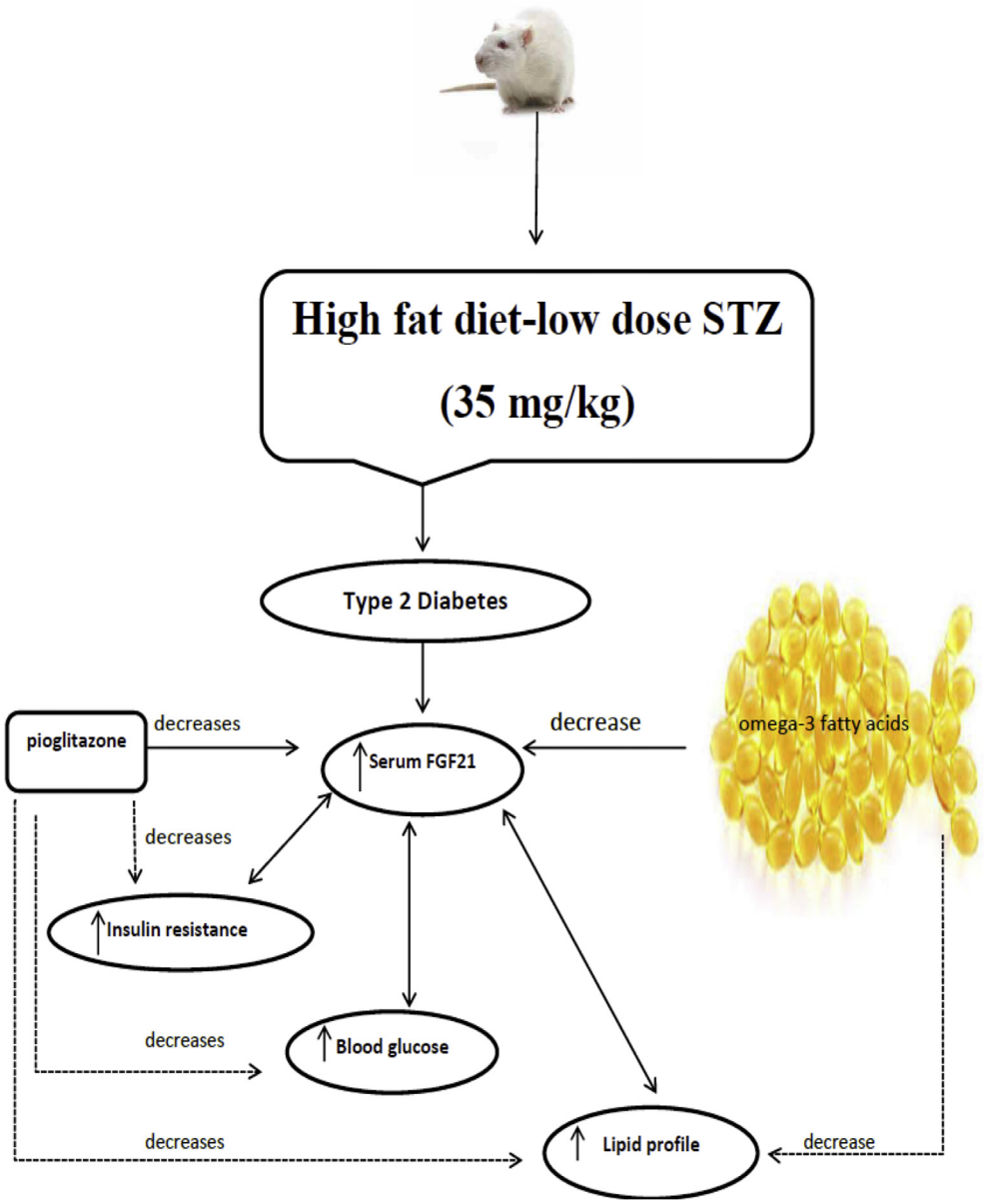

Fig. 7 – Lipid-lowering effect of ω-3 fatty acids and pioglitazone as well as blood glucose lowering and insulin-sensitizing effects of pioglitazone, mediated through FGF21.

diabetic group, while its hepatic concentration was decreased. This discrepancy may be due to the contribution of adipose tissue and other tissues in circulating FGF21 concentration. Hepatic mRNA expression of FGF21 showed significant decrease in diabetic group, which correlated positively to hepatic FGF21 concentration. Consistent with our results, Oishi and Tomita (2011) showed that pioglitazone significantly increased hepatic FGF21 mRNA expression in mouse liver and in cultured hepatocytes [62].

In agreement with our results, recent studies of Jin et al. (2014) and Lin et al. (2014) found that circulating FGF21 was significantly and positively correlated with fasting blood glucose concentration and HOMA-IR [63,64]. Also, we have found a significant positive correlation between serum FGF21 and both serum total cholesterol and triglycerides, which is consistent with Matuszek et al. (2010) [59].

6. Conclusion

Combining PPAR-α agonists, as ω-3 fatty acids, with PPAR-γ agonists, as pioglitazone, showed potential effects in lowering blood glucose concentration, improving lipid profile and insulin resistance. Such effects are mediated through modulation of FGF21 expression (Fig. 7).

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