Superparamagnetic Iron Oxide Nanoparticles in Type 2 Diabetes: A Mechanistic Study on Metabolic Pathway Regulation and Mitochondrial Function

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Abstract
Aim: Based on a previous study by our research group on superparamagnetic iron oxide nanoparticles (SPIONs) the current study aimed to explore the antidiabetic effects of one and two weekly doses of SPION-PEG-550 (22µmol Fe/Kg). The study focused on the metabolic pathways that may mediate the antidiabetic actions of SPIONs-PEG in peripheral tissues (skeletal muscle and kidney).
Methods: Diabetes was induced in 5-day neonatal rats with one dose of streptozotocin (70 mg/kg; n5-STZ rat model). At week 8, after confirmation of diabetes, rats were treated either with one or two weekly doses of SPION-PEG-550 (22µmol Fe/Kg) for 4 weeks. Fasting blood glucose level was monitored through the experiment. At the end of the study, rats underwent oral glucose tolerance test, serum samples were collected for biochemical analysis of insulin, adipocytokines, urea and creatinine. Muscles and kidneys were gathered to measure the gene expression of several metabolic pathways and the corresponding protein levels. Histopathology examination of muscle and pancreas was also performed.
Key finding: SPION-PEG-550 normalized the disturbed glucose homeostasis, reversed the insulin resistance, and adjusted the serum level of different adipocytokines. It modulated the insulin receptor substrates (IRS-1 and -2), improved several disturbed downstream effectors of the insulin signaling (AMPK and mTOR) and Wnt pathway. Besides, it normalized mitochondrial DNA copy number in both muscle and kidney. Histological examination of the muscle and pancreas has shown almost normal functional characteristics. Two doses of SPION-PEG-550/week have shown higher effectiveness but mild disturbed kidney function.
Significance: To our knowledge, this is the first study to examine the effect of SPIONs-PEG on signaling pathways in the muscle and kidney of diabetic rats.

KEYWORDS: Superparamagnetic iron oxide nanoparticles; SPIONs; Type 2 Diabetes; mtDNA; insulin resistance; WNT pathway.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder distinguished by hyperglycemia due to insulin deficiency. Normal insulin level is essential to facilitate glucose uptake into peripheral

tissues like the muscle, liver, and adipose tissue, hence, normalizing the blood glucose level [1]. Type 2 diabetes mellitus (T2DM) has shown a worldwide distribution reaching 462 million people with T2DM in 2017 and according to the Global Burden of Disease (Institute of Health Metrics, Seattle) database, its prevalence is increasing rapidly expected to reach 7079 million people with T2DM by the year 2030 [2]. Patients with chronic T2DM experience insulin resistance and the inability of the islet β cells to adequately use the secreted insulin levels [1]. Conventional treatment of T2DM includes synthetic oral hypoglycemic medication with/ without insulin, which is associated with poor patient compliance due to the daily dosing required and the cost [3]. Moreover, the typical treatment of T2DM with hypoglycemic agents has been challenging due to the heterogeneous mechanism of action, which has been associated with undesirable side effects [4].

In the last years, nanotechnology has gained more attention in the treatment of diabetes because of its ability to design carriers that selectively target specific tissues [5-7]. Nanoparticles as drug delivery carriers have shown a tendency to accumulate in the liver and kidney [8], causing also side effects that are related to the particle size of the Nano-carrier [9]. Superparamagnetic iron oxide nanoparticles (SPIONs) have shown unique properties such as high magnetization, stability, enhanced cellular absorption, and bio-distribution [10], without remarkable toxicity to the kidney or liver, unless used in very high doses [11]. Hence, SPIONs have been used as a diagnostic tool, drug delivery carrier, and cell separation utensil [11].

Recently, SPIONs itself, being the carrier of iron oxide, has shown effectiveness in the treatment of T2DM in a different context. Sharifi et al. has shown that SPIONs can downregulate genes involved in the pathophysiology of T2DM in human adipocytes [12]. Others have shown SPIONs to be effective carriers in erectile dysfunction associated with diabetic rats [13], as well as in the diabetes-related memory impairment experimental model [10]. Our research group has reported the effectiveness of SPIONs coated with the hydrophilic polymer, polyethylene glycol (PEG), on glucose homeostasis in the T2DM streptozotocin (STZ) animal model [14]. The anti-diabetic effect of SPIONs in this study was partially attributed to the lipotropic effect of SPIONs, as well as its effect on adipocytokines. Such anti-diabetic effect was dose-dependent; however, higher doses were associated with hepatorenal toxicities. The chain length of PEG has also been shown to affect the biodistribution of SPIONs, where the SPION-PEG-550 targeted the liver and SPION-PEG-2000 targeted the kidney [14].

Hence, the current study aimed to explore the antidiabetic effects different dose frequencies of 22µmol Fe/Kg SPION-PEG-550; one and two doses per week. The study focused on the metabolic pathways that may mediate the antidiabetic actions of SPIONs-PEG in peripheral tissues (skeletal muscle and kidney).

2. Materials and Methods

2.1 Preparation and characterization of SPIONs-PEG550:

The preparation and characterization of SPIONs-PEG550 Da were previously described by our collages [14]. Briefly, MeOPEG550-N($C_2H_4PO_3H_2$)$_2$ (194 mg, 0.25 mmol, Mw: 774 Da) was dissolved in 10 mL of the γ-Fe_2O_3 nanoparticles ferrofluid (γ-Fe_2O_3 original concentration was 4 g/L). After stirring for 10 minutes the pH was raised to 7.4 by dropwise addition of NaOH (1M). The colloidal suspension was stirred for 1 hour at room temperature, and then sonicated (4 min) and filtered through 0.45 and 0.22 µm nitrocellulose membrane filters (Millipore). Ferrofluid was purified by magnetic separation using a MidiMACS™ Separator and a LS column (MACS® Miltenyi Biotec), washed with NaCl 150 mM and finally collected in 10 mL of MilliQ water.

Iron content in the SPIONs suspension was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) in a plasma 40 ICP Perkin-Elmer spectrometer. Samples for ICP-OES were prepared by freeze drying the SPIONs suspensions and

digestion of the solid in concentrated HNO_3 overnight. Dynamic light scattering (DLS) and zeta potential measurements of ferrofluids were performed using a Malvern Zetasizer NS (Malvern Instruments Ltd., Worcestershire, UK) using a He–Ne laser with a 633 nm wavelength, a detector angle of 173° at 25 °C. Transmission electron microscopy (TEM) observations were carried out with a JEOL 2000-FXII microscope equipped with an Energy Dispersive X-ray (EDX) Microanalysis detector INCA. Infrared spectroscopy (FTIR) together with thermal analysis (TA) was performed to characterize the synthetized SPIONs and to confirm the attachment of the bis (Phosphonic) end capped PEG chains using in this work onto the iron oxide nanoparticles. FTIR and TGA experiments were performed on freeze dried samples purified by magnetic separation. Thermogravimetric (TGA) and Differential thermogravimetric (DTA) analysis were performed using a simultaneous DTA-TGA unit SDT-2960 (TA Instruments) in the range of 25ºC to 700ºC at a heating rate of 10ºC/min under air atmosphere.

2.2 Experimental animals:

A total number of 50 albino Sprague-Dawley male rats, 2 months old were used. The animals were purchased from the animal house facility of Medical Technology Center, Medical Research Institute, Alexandria University, Egypt. Rats were housed in standard cages in a well-ventilated room (25 ± 2 °C), with a relative humidity of (43 ± 3), with free access to water and food and 12 hours light/dark cycle. The animals were acclimated to the animal facility before the onset of the experiments. All procedures were performed in accordance with the Institutional Animal Care And Use Committee (IACUC)-Alexandria University, Egypt (Approval number: AU0122112432) The study also follows ARRIVE guidelines and comply with the National Research Council's guide for the care and use of laboratory animals.

- **Induction of the Neonatal Streptozotocin (n5-STZ) Rat Model of Diabetes**

Five 5 days old male Wistar albino pups (Pharos University, Alexandria, Egypt) were injected with freshly prepared STZ (90 mg/kg, i.p; Sigma, St. Louis, MO, USA,) in citrate buffer (pH 4.4). On day 21, the pups were weaned, and s were maintained on cafeteria diet (a mixture of chocolate, cookies and standard rat chow, containing approximately 57.7% carbohydrate, 19.5% protein and 22.8% fat by calories) to induce the n5-STZ type 2 model of diabetes [15]. Twelve weeks post induction the fasting blood glucose (FBG) level was measured by Glucometer (ACCU CHEK Active, Roche Co.), rats with fasting blood glucose level higher than 200 mg/dl were considered diabetic and were included in the study. All rats had free access to water and food with 12:12 h light/dark cycle, normal humidity, and good sanitary constant environmental conditions prior to experimentation and thereafter.

2.3 Experimental design

Animals were classified into the following groups (6 rats each): 1) Control healthy group, 2) untreated-diabetic group, 3) Metformin-treated diabetic group that was orally treated with metformin in a dose of 200 mg/kg/day for four weeks [16], 4) one dose SPION-PEG-treated diabetic group, in which diabetic rats were intravenously injected with SPION-PEG at a dose of 22µmol Fe/kg once a week for four weeks [14] and 5) two dose SPION-PEG treated diabetic group, in which diabetic rats were intravenously injected with SPION-PEG at a dose of 22µmol Fe/kg twice per week for four weeks.

2.4 Collection of biological samples

At the end of the experiment time, oral glucose tolerance test [20] was carried out for all groups. Then, animals in all studied groups were overnight fasting, then were anesthetized and serum samples were prepared by collecting the blood from the retro-orbital followed by centrifugation at 3000×g for 10 min. The obtained serum was used to determine serum glucose, insulin, urea, and creatinine levels. Then animals were sacrificed through cervical dislocation to obtain muscle and kidney tissues for the assessment of mitochondria DNA

copy number (mtDNA-CN), gene expression and protein levels of IRS1, IRS2, AMPK, mTOR, WNT3A, GSK3β, β-catenin, and FOXO1. Also, the renal expression of Kim1 and lipocalin-2 at mRNA level (as markers of the renal damage) was assessed.

2.5 Methods:

2.5.1 Oral blood glucose tolerance test (OGTT): was carried out after overnight fasting, a blood droplet was collected from the tail vein directly onto the test strip (baseline) and glucose solution was then administered by oral gavage at a dose of 2.5 g/Kg. Blood was collected at 30, 60, 90 and 120 min after glucose administration. Glucose level was determined using Glucometer (ACCU CHEK Active, Roche Co.).

2.5.2 Routine parameters: Fasting blood sugar (FBS) level, serum urea, creatinine, triglycerides (TG), total cholesterol (TC) and high-density lipoprotein-cholesterol (HDL-C) levels were determined following the instructions of the reagents obtained from BioMed Diagnostics INC (USA). Serum low density lipoprotein-cholesterol (LDL-C) was calculated from TG, TC and HDL-C concentrations using the following equation:

$$\text{LDL-C (mg/dL)} = TC - (HDL\text{-}C) - TG/5$$

2.5.3 Serum insulin and adiponectin:

Fasting insulin level was determined using Insulin rat ELISA kit (EMD Millipore, USA) according to the manufacturer instructions.

2.5.4 Tissues protein contents using ELISA:

The protein contents of insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) were assayed using rat ELISA kits obtained from Chongqing Biospes (China, Cat. no. BYEK2344 and BYEK2345). The protein level of phosphorylated-AMP kinase (p-AMPK, active from) was assayed using competitive ELISA kit (MyBiosource, USA, cat.no. MBS7230575). The active phosphorylated-mammalian target of rapamycin at Ser 2448 (p-mTOR) was assayed using ELISA kit obtained (abcam, USA, cat. no. ab168538). The inactive phosphorylated-Glycogen synthase kinase-3β at Ser9 (p-GSK-3β) was assayed using Duoset@IC ELISA kit (R&D system, USA, cat. no. DYC1590). The wingless-Type MMTV Integration Site Family, Member 3A(WNT3A) was assayed by using rat ELISA kit obtained from LSBIO, USA (cat. no. LS-F8574). The protein content of β-catenin was assayed using Enzo ELISA kit, USA (cat. no. ADI-900-135), and Forkhead Box O1 (FOXO1) was assayed using ELISA kit obtained from MyBiosource, USA (cat.no. MBS266917). All the procedures were performed according the manufacturer's instructions

2.5.5 Gene expression analysis using RT-PCR: The quantitative analysis of IRS-1, IRS-2, AMPK, mTOR. WNT3A, GSK-3β, β-catenin, and FOXO1 genes expression at mRNA level in muscle and kidney tissues and Kim-1 and lipocalin-2 in kidney tissue was performed using quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR). The total RNA was extracted from the studied tissue RNeasy Mini Kit (Qiagen,Germany) according to the manufacturer instructions. The reverse transcription of the extracted RNA was done using MiScript II RT Kit (Qiagen, Germany) according to the manufacturer instructions using miScript HiFlex buffer. The obtained cDNA then amplified and detected using specific primers for IRS-1, IRS-2, AMPK, mTOR. WNT3A, GSK-3β, β-catenin, FOXO1, Kim-1, and lipocalin-2 (Table 1) by qRT-PCR assay using Rotor-Gene SYBR Green PCR Kit (Qiagen, Germany). Rotor-Gene Q-Pure Detection version 2.1.0 (build 9) (Qiagen, Valencia, CA, USA) was used to determine the Ct values. For each gene, the expression was calculated relative to 18SrRNA as a reference gene using the formula: $2^{-\Delta\Delta Ct}$.

2.5.6 Mitochondrial DNA (mtDNA) copy number

Total DNA was extracted from muscle tissues using mini DNeasy kit (Qiagen Germany). In the extracted total DNA, the mtDNA specific sequence was assessed relative to the nuclear DNA specific gene (PGC1α) using qPCR [17]. The primers used were presented in Table (1). PCR reactions were carried out using SYBR Green PCR Master Mix (Qiagen, Germany), 0.5 µM forward and reverse primer, and 50 ng genomic DNA were used with the following conditions: 95˚C for 10 min followed by 40 cycles of 95˚C for 15

sec, 60˚C for 30 sec and 72˚C for 30 sec. The relative mtDNA copy number was calculated using the $2^{-\Delta\Delta Ct}$ method as described previously [17].

2.5.7 Histological analysis of muscle and pancreas

Morphological examination of the pancreas of SPION-PEG-550 treated diabetic rats have shown clear improvement compared to diabetic untreated ones, revealing normal islets with only some focal areas of cellular infiltration. Examination of the gastrocnemius muscle came in favor of the two-dose regimen of SPION-PEG-550, where the muscle fibers appeared almost normal. The one-dose regimen of SPION-PEG-550 has also shown improvement in the muscle structure compared to diabetic untreated rats.

2.5.8 Statistical analysis:

Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). The data were expressed as mean±SD. Comparisons between different groups were made using one way ANOVA followed by Tukey post-hoc test.

3. Results

3.1 Glucose Homeostasis parameters:

- **Oral glucose tolerance test (OGTT)**

The fasting blood glucose level of the diabetic rats is significantly higher than control rats by about 2-folds. Normally, half an hour after oral administration of glucose, the glucose level reached its peak then decreased gradually to near the fasting level after 120 minutes. However, in the diabetic rats, the blood glucose was elevated by about 223% above the normal value after 30 minutes and failed to decline significantly even after 2 hours, confirming a state of impaired glucose tolerance (IGT) **(Table 2, Figure 1)**. The diabetic rats treated with different treatment regimens significantly abated the high glucose levels at the time intervals during OGTT with the best effects observed in the rats treated with metformin and SPIONs at 2 doses per week which confirmed by the AUC (**Figure 1).**

- **Fasting blood glucose and insulin levels and HOMA-IR:**

As indicated previously during OGTT, the untreated diabetic rats have a significant elevation in FBS and insulin level compared to control rats. This results in a significant elevation in HOMA-IR as indicated in Table 3. The treatment of the diabetic rats with metformin or SPIONs (either one or two doses per week) significantly corrected the level of FBS and HOMA-IR while insulin level still higher than the control values. No significant differences in FBS were observed between the diabetic rats treated with metformin and SPIONs while the insulin level showed a significantly lower level in metformin-treated diabetic rats compared to SPIONs treated diabetic rats (Table 3).

3.2 Serum urea and creatinine:

As presented in **Table (3)**, the serum urea levels showed no significant differences between the studies groups. The creatinine level showed mild significant elevation in the untreated diabetic rats, while the diabetic rats treated with metformin or SPIONs at one dose per week have significantly lower levels compared to the untreated rats. The diabetic rats treated with SPIONs at two doses per week have significantly higher creatinine levels compared to the control group and the diabetic group treated with metformin.

3.3 Gene expression of insulin receptors:

In both tissues, the IRS1 gene expression at mRNA and protein levels was significantly downregulated in the untreated diabetic rats compared to control rats

(Figure 2A and 2B). In muscle tissue, the expression of IRS1 at mRNA level showed no significant difference in the metformin and SPIONs treated diabetic rats compared to the untreated diabetic rats whereas, the rats treated with SPIONs one dose per week showed significantly lower IRS1 compared to the control rats. At the protein level, IRS1 in muscle showed a non-significant increase in the diabetic rats treated with metformin and one dose SPIONs per week whereas the diabetic rats treated with two doses SPIONs per week showed significant higher IRS1 protein level compared to the untreated diabetic rats (Figure 2A and 2B). In the kidney, the treatment of diabetic rats with metformin or SPIONs significantly normalized the expression of IRS1 at mRNA level compared to the untreated diabetic rats. At the protein level, the treatment with metformin and two doses of SPIONs per week significantly increased IRS1 level compared to the untreated rats but still significantly lower than the control level. The treatment with one dose of SPIONs per week lacks a significant effect on IRS1 protein compared to the untreated rats (Figure 2A and 2B).

In muscle, the expression IRS2 at mRNA and protein levels showed no significant changes between the studied groups. In the kidney, the expression IRS2 at mRNA level showed significant upregulation in all diabetic rats compared to the control rats. At the protein level, the untreated diabetic rats showed significantly higher IRS2 levels compared to the control group. The diabetic rats treated with metformin or SPIONs (one or two doses per week) showed significantly lower IRS2 protein compared to the untreated rats and significantly higher levels than the control group (Figure 2A and 2B).

3.4 Serum adipocytokines:

The diabetic rats have significantly higher serum levels of leptin and TNF-α and significantly lower level of adiponectin compared with the control rats. The diabetic rats treated with metformin lower level of leptin and TNF-α, and higher level of adiponectin compared with the untreated rats. The diabetic rats treated with SPIONs (especially the rats treated with two doses per week) have significantly corrected levels of leptin, TNF-α, and adiponectin compared with untreated rats especially the rats. The treatment with two doses of SPIONs per week have similar effects on TNF-α, and adiponectin compared with metformin but have significantly better effect on leptin level (Table 4).

3.5 Gene expression of AMPK and mTOR

At mRNA level, the expression of AMPK showed no significant changes between the studied groups in muscle and kidney tissues (Figure 3A). At the protein level, the active p-AMPK contents were significantly lower in the muscle and kidney tissues of untreated diabetic rats compared to the control rats. In muscle, only the diabetic rats treated with two doses of SPIONs per week have significantly higher p-AMPK content compared to the untreated rats. In the kidney, all diabetic rats treated with metformin or SPIONs have significantly higher p-AMPK content compared to the untreated rats (Figure 3B).

Regarding mTOR expression at mRNA, the untreated diabetic rats have significantly higher expression in muscle and kidney tissues compared to control. In muscle, all the treated diabetic rats have significantly lower AMPK expression compared to the untreated rats but significantly higher the control rats. In the kidney, all the treated diabetic rats have significantly higher expression levels compared to control rats with no significant changes compared to the untreated rats (Figure 3A). At the protein level, the active phosphorylated mTOR (p-mTOR) contents were significantly higher in the muscle and kidney tissues of untreated

diabetic rats compared to the control rats. In muscle, the diabetic rats treated with metformin or SPIONs have significantly lower p-mTOR contents compared to the untreated rats but significantly higher the control. In the kidney, only the diabetic rats treated with SPIONs (one or two doses per week) have significantly lower p-mTOR content compared to the untreated rats (Figure 3B).

3.6 Expression of WNT3a and GSK3B at mRNA and protein levels.

The expression of WNT3a in muscles and kidney tissues is upregulated at mRNA level in the untreated diabetic rats compared to control rats (Figure 4A). In muscle, the treatment of diabetic rats with metformin or SPIONs have no significant effects on the expression of WNT3a. In the kidney, the diabetic rats treated with metformin and SPIONs have significantly lower expression levels compared to the untreated rats with the best effect observed in the diabetic rats treated with SPIONs (one or two doses per week) which have significantly lower expression level compared to the rats treated with metformin (Figure 4A). At the protein level, the muscle and kidney tissues contents of WNT3a are significantly higher in the untreated diabetic rats. The treatments of diabetic rats with metformin or SPIONs lack significant effects on the WNT3a protein level in muscle and kidney tissues (Figure 4B).

At mRNA level, the expression of GSK3B is significantly upregulated in the muscle and kidney tissues of untreated diabetic rats. The treatment of diabetic rats with metformin or SPIONs significantly normalizes the expression of GSK3B in both muscle and kidney tissues (Figure 4A). At the protein level, the phosphorylated glycogen synthase kinase-3β at serine 9 (p-GSK-3β); the inactive form, is significantly higher in the muscle and kidney tissues of the untreated diabetic rats. Only the diabetic rats treated with two doses of SPIONs per week have significantly lower p-GSK3B in the muscle tissues compared to the untreated rats. In the kidney, no significant changes in the level of p-GSK3B occur in all treated rats compared to the untreated rats (Figure 4B).

3.7 Expression of β- catenin and FOXO1 at mRNA and protein levels

The expression of β-catenin at mRNA and protein levels in the muscle tissues of all diabetic rats (untreated or treated) shows no significant changes compared to the control rats. Only the diabetic rats treated with two doses of SPIONs per week have significant upregulation in β-catenin expression at mRNA level (Figure 5A and B). In the kidney, the untreated diabetic rats have a significantly lower expression level of β-catenin at mRNA and protein levels compared to the control rats. The diabetic rats treated with SIPONs (one or two doses per week) have significantly higher expression level at mRNA level compared to the untreated rats with the best effect observed in the rats treated with two doses of SPIONs per week which have significantly higher β-catenin expression compared to the rats treated with metformin. At the protein level, all the treated diabetic rats have a significantly higher level of β-catenin protein compared to the untreated diabetic rats but still significantly lower than the control value (Figure 5A and B).

At mRNA level, the expression of FOXO1 showed significant upregulation in both muscle and kidney tissues of the untreated diabetic rats compared to the control rats. The treated diabetic rats showed significant downregulation of FOXO1 in muscle and kidney tissues compared to the untreated rats but the levels still significantly higher than the control values with the best effect observed in the rats treated with two doses of SIONs per week (Figure 5A). At the protein

level, the untreated diabetic rats have a significantly higher expression level of FOXO1 in the muscle and kidney tissues compared to control. The treatment of diabetic rats with metformin or SPIONs significantly normalizes the FOXO1 protein in muscle. In kidney tissue, metformin treatment lacks a significant effect on the FOXO1 protein compared to the untreated rats while SPIONs treatment significantly declines the FOXO1 protein compared to the untreated rats and completely normalized using two doses of SPIONs per week (Figure 5B).

3.8 Kidney expression of Kim-1 and lipocalin-2 genes

The renal expression of Kim-1 and Lipocalin-2, as molecular markers of renal damage, are significantly upregulated in the untreated diabetic rats. The expression of lipocalin-2 significantly downregulated to a similar extent in the diabetic rats treated with metformin or SPIONs (one or two doses per week). While the rats treated with metformin and one dose of SPIONs per week have significantly lower Kim-1 expression levels compared to the untreated diabetic rats, the rats treated with two doses of SPIONs per week doses do not have a significant change in Kim-1 expression compared to the untreated rats. The diabetic rats treated with SPIONs have significantly higher expression levels compared to the diabetic rats treated with metformin (Figure 6).

3.9 Mitochondrial DNA (mtDNA) copy number as a marker of mitochondrial biogenesis.

The muscle and kidney tissues content of mtDNA assayed as mtDNA copy number per diploid cell are significantly declined in the untreated diabetic rats, especially in the muscle, compared to the control rats. The treatment of diabetic rats with metformin lacks significant correction on the mtDNA copy number in both tissues. The diabetic rats treated with SPIONs have a significant elevation of mtDNA copy number in both tissues compared to the untreated rats and completely normalized in the rats treated with two doses of SPIONs per week (Figure 7).

3.10 Histopathology of pancreas and gastrocnemius muscle

Morphological examination of the pancreas of SPION-PEG-550 treated diabetic rats have shown clear improvement compared to diabetic untreated ones, revealing normal islets with only some focal areas of cellular infiltration. Examination of the gastrocnemius muscle came in favor of the two-dose regimen of SPION-PEG-550, where the muscle fibers appeared almost normal. The one-dose regimen of SPION-PEG-550 has also shown improvement in the muscle structure compared to diabetic untreated rats.

4. Discussion

The present study confirms the antidiabetic effects of SPIONs and provides evidence of the crosstalk between different signaling pathways in the muscles and kidney in mediating these effects. Based on our previous study on SPIONs-PEG-550 and SPIONs-PEG-2000 and the associated side effects of the high doses used (44 and 66 µmol Fe/Kg) [14], the current study focused on the antidiabetic potential of low dose SPIONs-PEG-550 (22 µmol Fe/Kg) used at two frequencies throughout the week (one and two doses per week) aiming to reach better therapeutic effect with low or no toxicity. The different metabolic signaling pathway in the muscle and kidney of diabetic rats were also spotlighted.

SPIONs-PEG-550 treatment significantly normalized the blood sugar level and decreased the insulin resistance, an effect that was comparable to the metformin. This has been confirmed

by OGTT, where both dose regimen of SPIONs have shown correction of glucose tolerance in diabetic rats, beside the near normal histological appearance of the pancreas of SPIONs treated rats. The effect of metformin on blood glucose and insulin resistance has been well established in earlier studies [18, 19]. Matching the results of our previous study, SPION-PEG-550 at 22µmol Fe/kg has shown lower serum insulin level and HOMA-IR than untreated diabetic rats. However, the serum insulin level was significantly higher than metformin. This in turn suggests that, like metformin, the efficacy of SPIONS requires the presence of insulin; in addition, it might enhance insulin sensitivity.

Although iron overload is always associated with increased insulin resistance especially in adipocytes [20, 21], superparamagnetic iron oxide nanoparticles seem to be devoid of such adverse effect. This has been confirmed by the results of the serum adipocytokines in this study. The diabetic rats suffered from significant decline in the serum level of adiponectin which considered as a marker of insulin sensitivity and positively correlated with insulin sensitivity and inversely correlated with type 2 diabetes risk [22]. SPIONs significantly increased adiponectin levels to similar extend as metformin especially when used at two doses per week which may contribute at least partially to the glucose-lowering effect of SPIONs. Also, SPIONs significantly decreased the elevated levels of leptin and TNF-α level in the diabetic rats. The effect of SPIONs on leptin level is significantly better than the effect of metformin which may imply a leptin-sensitizing effect of SPIONs. The fact that decreased leptin secretion is associated with diminished pro-inflammatory cytokines level, decreased insulin resistance and protection from type 2 diabetes [23], support the current results of HOMA-IR and low blood sugar level. This besides adiponectin, which possesses anti-inflammatory and insulin-sensitizing properties, playing an important role in the metabolism of glucose and lipids [22, 24].

High TNF-α levels in diabetes stimulate IKKβ/NF-κB pathway, which blocks the insulin receptor substrate (IRS) leading to insulin resistance [25]. This in turn explains the low mRNA and protein expression levels of IRS1 in the muscle and kidney of diabetic rats in the current study. Comparable to metformin, SPIONs administration has shown increased protein levels of IRS1 in the muscle, without affecting its gene expression that may indicated that SPIONs may affect the rats of IRS1 stability, degradation and/or activation rather than induction at mRNA level. Metformin being an insulin-sensitizing medication, its action depends on improving peripheral tissue insulin sensitivity, especially in skeletal muscles and adipocytes [26, 27]. Its mechanism has been further clarified by Kumar and Dey [28], who proved that metformin can enhance insulin signaling in skeletal muscles by enhancing tyrosine phosphorylation of IRS1. This in turn explains its effect on IRS1 protein level in the muscle of the current study and hence enhancing the metabolic action of IRS associated phosphatidylinositol 3-kinase (PI3K)/AKT pathway [29].

Insulin receptors are expressed on cell types of the kidney including podocytes and tubular cells [30]. Hyperinsulinemia and insulin resistance cause decreased insulin-mediated nitric oxide generation affecting the glomerular filtration rate, beside stimulating renal sodium transport causing hypertension [31]. This explains the decrease in IRS1 expression and protein level in the kidney of diabetic rats, which has been reversed by metformin and SPION. In the last few years, several studies have focused on the beneficial effect of metformin in kidney failure [32-34]. Interestingly, IRS1 and IRS2 expression in the kidney of diabetic rats have shown to be inversely proportional, and hence their protein levels. Such effect has been observed previously in hepatocytes with and without insulin stimulation during fasting-feeding transition [35]. High IRS2 expression in diabetic nephropathy has been reported earlier by Hookham et al.[36] and Carew et al. [37], highlighting the role of insulin receptors for normal kidney function. Upregulation of IRS2 in diabetic kidney was considered protective mechanism to prevent damage of renal cells by glucose and induce

insulin-related cell survival [36]. The complementary effect of IRS1 and IRS2 is then essential for the renal response to insulin and the insulin downstream signaling. Our data suggest that metformin, as well as SPIONs, are essential modulators of the IRS1/IRS2 expression and they may be also responsible for sensitizing the kidney to insulin. As a result, we propose that this sensitization acts as a feedback signal to regulate the level of insulin produced.

To our knowledge this is the first study to examine the effect of SPIONS on the expression of IRS1 and IRS2 in the muscle and kidney, highlighting its effect on IRS1 protein level in the muscle, as well as affecting the expression and protein level of IRS2 in the kidney.

The PI3K/AKT arm of the insulin signaling pathway is a central node in metabolic regulation, where its activation regulates several downstream effectors as AMP-activated protein kinase (AMPK), mTOR complex 1 (mTORC1), glycogen synthase kinase 3 (GSK3) and forkhead box O family members (FOXO). Research shows a direct feedback inhibition of IRS signaling by mTOR, where activation of mTOR affects the phosphorylation of IRS1 and IRS2, but not their expression level [38, 39], leading to their degradation and loss of function [40]. This explains the high mTOR levels in the muscle and kidney of diabetic rats in this study and the associated decrease in IRS1; however, the IRS2 levels don't seem affected by the change of mTOR levels in the current study. As stated by Long et al. [41] IRS1, but not IRS2, deficiency could decrease skeletal muscle mass and has the main role of insulin- like signaling in skeletal muscle. The study by Kido et al. [42] also suggested that mTOR/IRS1 signaling inhibition was sufficient to enhance insulin sensitivity in skeletal muscle. Drugs that inhibit mTOR activity are able to relieve the mTOR- dependent negative feedback inhibition, allowing the IRS1 signaling pathway to take place [43]. The role of metformin in inhibiting mTOR in muscle and kidney has been previously explored [44-47]. SPIONs administration in this study showed significant downregulation of mTOR expression at mRNA and protein levels in the muscle and kidney tissues. Iron oxide nanoparticles have shown a cross-talk with mTOR signaling in many contexts [48, 49], however; the effect of SPIONS on diabetic muscle or kidney through mTOR signaling is considered novel approach in the current study.

Normal insulin signaling activates the Akt-mTOR pathway, inhibits FOXO and limits AMPK activity [41]. Activation of AMPK (by phosphorylation) is related to prolonged starvation, increased AMP/ATP ratio or depleted glycogen, which in turn stimulate the catabolic pathway including fatty acid oxidation to generate energy in skeletal muscles. On the other hand, it suppresses the anabolic pathway, decreasing mTOR/Raptor protein synthesis and hence the muscle mass [50]. In type 2 Diabetes, p-AMPK (active form) has shown to increase insulin sensitivity and improve the metabolic pathway [51]. In the last years, metformin activated AMPK was associated with activation of IRS1, inhibition of the PI3K/AKT pathway [43], anti-hyperglycemia and antitumor effects [52-54], as well as protection against renal damage [55]. Similarly, SPIONS treatment in the current study successfully corrected diabetes-induced decrease in p-AMPK protein in muscle and kidney, where the higher dose regimen of SPIONS has shown the most prominent effect.

FOXO1 action is inhibited by insulin through phosphorylation [56], while oxidative stress induces the activation of FOXO signaling [57]. Also, FOXO activity is regulated by AMPK and has important role during fasting. High expression of FOXO1 activates autophagy related proteolysis, promotes muscle atrophy and renal failure [58, 59]. FOXO1 is considered as a negative regulator of skeletal muscle mass and type 1 muscle fiber-related genes and it act through interaction and binding with peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 α (PGC-1α) that might inhibit its function [60]. PGC-1α is a major regulator of oxidative metabolism by playing a key role in regulating mitochondrial gene expression and mitochondrial biogenesis [61]. The previous study indicated that

SPIONs administration enhanced the expression of PGC-1α in a dose-dependent manner in rat model of T2DM [14]. This effect combined with the FOXO1-lowering effect of SPIONs may enhance the biological activity of PGC-1α that may mediates the ameliorative effect of SPIONs on mtDNA copy number as a biomarker of mitochondrial biogenesis as documented in the present work.

Our results show that SPIONS, like metformin, was capable to suppress FOXO1 transcription, as well as protein level in muscle and kidney of diabetic rats. One of the mechanisms of action of metformin has shown to be FOXO1 inhibition in cases of diabetes related kidney injury [62] as well as in other disease models [63-65].

Another important signaling pathway that play key role in the pathogenesis of T2DM and its complications in muscles and kidney is Wnt pathway [57]. In the absence of WNT stimulation, β-catenin is located within the 'destructive complex' and phosphorylated by GSK-3β resulting in its proteasomal degradation. Following WNT stimulation, the destructive complex disassembles resulting in accumulation of free β-catenin that enters the nucleus and forms the bipartite transcription factor β-catenin/TCF, leading to enhanced expression of the target genes [66]. In the current study, induction of diabetes caused marked increase in the expression of WNT3A at mRNA and protein levels in muscle and kidney tissues, these increases were associated with significant decline in the expression of β-catenin (at mRNA and protein levels) and significant decrease in the inactive phosphorylated-GSK3β at Ser9 (p-GSK-3β). Despite the increased level of WNT3A, the low β-catenin and low inactive p-GSK3β (high active GSK-3β as documented in the previous study [14] resulted in impairment of WNT pathway in muscle and kidney. Overexpression of Wnt1 and Wnt5a in developing pancreas of a former study by Heller et al. [67] disrupted normal pancreas development, causing reduction in islet formation and associated insulin resistance. Overexpression of GSK-3β in β-cells of mice was associated with decreased β-cells mass, proliferation and hence glucose tolerance [68]. Increased IRS2 expression and protein level in this study were positively correlated with Wnt3a levels, an effect that appeared in the kidney, but not the muscle. This outcome matches the previous study by Carew et al. [37], who suggested that IRS2 deletion in the kidney leads to reduced GSK-3β activity and consequently β-catenin accumulation. Treatment with metformin, as well as SPIONs could not correct all aspects of the WNT-signaling pathway observed in diabetic rats in the current study. However, the two-dose regimen of SPIONS has shown to be superior in effect to metformin in some of muscle and kidney parameters. The observed enhancement of β-catenin expression in the kidney of treated rats was associated with decreased expression of FOXO1, this outcome supports the fact that β-catenin can bind to FOXO transcription factors and enhance the transcription of its regulating genes [69]. The ability of metformin to activate the Wnt/ β-catenin pathway has been observed in many other contexts, and its inhibition of GSK-3β has been related to AMPK activation [70-72].

The most distinct effect of SPIONs in the present study is the effect on mtDNA-CN in the muscle and kidney. SPIONs treatment shows prominent enhancement of the tissues mitochondrial biogenesis assayed as mtDNA-CN which returned to the normal values in the treated diabetic rats, an effect that was not observed upon treatment with metformin. As increased IRS2 levels in peripheral tissues increase oxidative stress and may cause mitochondrial dysfunction [73], that may explains our results in the kidney of diabetic rats.

The decreased mtDNA-CN could impair the function and metabolism in the muscle and kidney tissues and may be considered the prime event in the development of insulin resistance [74]. Many studies relate the reduced mitochondria in the peripheral tissues to the pathogenesis of T2DM [74-76]. So, the reduced mtDNA number and function results in impaired beta-oxidation of fatty acids that would result in enhanced efflux of fatty acid into circulation causing impaired insulin sensitivity in the peripheral tissues [77].

Decreased mtDNA-CN is believed to be followed by feedback increase in FOXO1 levels as a compensatory mechanism to resist the oxidative stress [59], which explains the results seen in the diabetic untreated rats of the current study. The enhancing effect of SPIONs on mtDNA-CN was dose related; and histological examination of the muscle came in favor of the two-dose per week regimen of SPION-PEG-550 showing almost normal muscle fibers.

The mechanism(s) of action SPIONs-PEG550 in muscle and kidney tissues are unclear and need further investigations. Both moiety of SPIONs-PEG550; SPIONs and PEG, may participate in the observed actions in diabetic rats. PEG moiety facilitates transport across membranes and penetration into intracellular spaces and mitochondria and allows distribution into distant tissues (e.g., leg muscles) after intraperitoneal injection and exert significant physiologic effects on these distant organs [77].

Although, the potential benefits of SPIONs-PEG on the muscle and kidney tissues of the diabetic rats, there is a distinct need to identify any potential cellular damage associated with its applications. The dose used in the present study (22µmol Fe/Kg) does not aggravates the kidney function tests compared with the untreated rats. At the molecular level, the kidney expression of kidney injury molecule-1 (Kim-1) and lipocalin-2 showed marked upregulation in the untreated diabetic rats. Both are considered as a more sensitive biomarker than traditional biomarkers such as creatinine and urea [78]. Their expression appears early during renal damage and detect the injury before the start of any histopathological alterations [79, 80]. The expression of lipocalin-2 significantly downregulated to a similar extent in the diabetic rats treated with metformin or SPIONs (one or two doses per week), while Kim-1 expression significantly downregulated in the rats treated with metformin and one dose of SPIONs per week. The rats treated with two doses of SPIONs per week doses do not have a significant change in Kim-1 expression compared with the untreated rats. This may indicated that, the two-dose regimen of SPION-PEG-550 may lacks protective effects against diabetes-induced renal damage but does not induce further renal damage.

5. Conclusion

In conclusion, SPION-PEG-550 was able to normalize the disturbed glucose homeostasis, reverse the insulin resistance and adjust the serum level of different adipocytokines. In the kidney, it modulated the IRS1/IRS2 levels. Besides, it improved the disturbed Wnt3a, GSK3β, AMPK, mTOR, β-catenin and FOXO1 signaling in both muscle and kidney. Administration of SPIONS once or twice per week remarkably increased the mtDNA expression in both muscle and kidney, highlighting the role of the mitochondria in the SPIONS mechanistic pathway. Histological examination of the muscle and pancreas has shown almost normal functional characteristics. Two dose administration of SPION-PEG-550 per week has shown higher effectiveness in most studied parameters without adverse effects on the kidney.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure caption

Figure 1: Oral glucose tolerance test in control rats, untreated diabetic rats, metformin-treated diabetic rats, and diabetic rats treated with one and two doses of SPIONs. (Data presented as Mean ± SD, N=10. a Significantly different from control rats, and [b] Significantly different from untreated diabetic rats by ANOVA ($p<0.05$))

Figure 2: The expression of IRS1 and IRS2 at mRNA (A) and protein level (B) in muscle and kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, and [b] Significantly different from untreated diabetic rats by ANOVA ($p<0.05$))

Figure 3: The expression of AMPK and mTOR at mRNA (A) and protein level (B) in muscle and kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, and [b] Significantly different from untreated diabetic rats by ANOVA ($p<0.05$))

Figure 4: The expression of WNT3a and GSK3B at mRNA (A) and protein level (B) in muscle and kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, [b] Significantly different from untreated diabetic rat, and [c] Significantly different from metformin-treated rats by ANOVA ($p<0.05$))

Figure 5: The expression of β-catenin and FOXO1 at mRNA (A) and protein level (B) in muscle and kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, [b] Significantly different from untreated diabetic rat, and [c] Significantly different from metformin-treated rats by ANOVA ($p<0.05$))

Figure 6: The expression of Kim-1 and lipocalin-2 in kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, [b] Significantly different from untreated diabetic rat, [c] Significantly different from metformin-treated rats, [d] Significantly different from rats treated with SPIONs one dose per week, by ANOVA ($p<0.05$))

Figure 7: The mitochondrial DNA (mtDNA) copy number in muscle and kidney tissues of control rats and diabetic untreated or treated with metformin or SPIONs at one dose or two doses per week. (Data presented as Mean ± SD, n = 10. [a] Significantly different from control rats, [b] Significantly different from untreated diabetic rat , and [c] Significantly different from metformin-treated rats, by ANOVA ($p<0.05$))

Figure 8: Histopathology examination of pancreas and gastrocnemius muscle. (A) Pancreas of diabetic rat showing vascular congestion. Cells of islet appear degenerated with dark nuclei& vacuolated cytoplasm. (B & C) Diabetic pancreas treated with one dose of SPION-PEG-550 per week showing focal area of cellular infiltration (arrow) with normal appearance of islets. (D & E) Diabetic pancreas treated with two doses of SPION-PEG-550 per week showing restored normal pancreas architecture with evident lobulation. Pancreatic acini appear normal. Islets of Langerhans appear formed of normal cells. (F) Gastrocnemius muscle from diabetic rat showing evident vascular congestion with cellular infiltration. Muscle fiber appear pale eosinophilic. (G & H) Gastrocnemius muscle from diabetic rat treated with one dose of SPION-PEG-550 per week showing focal area of vascular congestion. Some muscle fibers appear pale eosinophilic (decreased myofilaments). (I & J) Gastrocnemius muscle from diabetic rat treated with two doses of SPION-PEG-550 per week showing regularly oriented muscle fibers with apparent eosinophilia.

Figure 9: Spion-PEG-550 affects the cross-talk between insulin signaling, Wnt pathway and mitochondria. Black arrows indicate activation/upregulation and blunt ends (green) indicate suppression/downregulation in normal tissue. Pathways enhanced or inhibited by SPION-PEG-550 are presented as red arrows. IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; mTOR, mechanistic target of rapamycin; FOXO1, forkhead box O family member 1; AMPK, AMP-activated protein kinase; GSK3, glycogen synthase kinase 3; Dsh, dishevelled protein; mtDNA, mitochondrial DNA; LRP, low-density-lipoprotein-related protein