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ORIGINAL ARTICLE

Omentin-1 a Novel Approach Ameliorates Glycemic State, Inflammation and Osteopontin Level in GDM Rat Model: PI3K/AKT/GSK-3 pathway.

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ABSTRACT

Background: Omentin-1 an adipokine expressed in visceral fat, endothelium and gut. Its level was found lower in obesity and type II diabetes. It is expressed in placenta and was associated with insulin sensitivity during pregnancy.

Methods: Rats were divided into 4 groups; group I: control, group II: sham group (normal pregnant), group III: gestational diabetic group and group IV: omentin-1 treated gestational diabetic group. Blood glucose, insulin HOMA-IR, lipid profile, CRP and osteopontin (OPN) levels were measured and gene expression of PI3K, AKT, GSK-3, NF-κB and OPN

Results: In group III, a significant increase in insulin resistance, CRP, OPN, increased mRNA expression of GSK-3, NF-κB, and OPN while PI3K and AKT expression decreased. Omentin-1 treatment in group IV attenuated insulin resistance, increased PI3K and AKT expression but decreased OPN, GSK-3, and NF-κB expression.

Conclusion: Omentin-1 administration in gestational diabetic rats improved insulin sensitivity and attenuated inflammatory response through PI3K/AKT/GSK-3 pathway.

Keywords: omentin-1, osteopontin, gestational diabetes, PI3K.

INTRODUCTION

Gestational diabetes mellitus (GDM) affects 1 to 30% of pregnant women worldwide. It is referred to as a temporal disruption in carbohydrates metabolism during pregnancy. Overweight and obesity in mothers are prominent risk factors [1]. It is believed that central mediators of this increased insulin resistance are pro-inflammatory cytokines [2]. Omentin-1, also called intelectin-1, is an adipokine that is mostly expressed in visceral fat as well as vascular cells, gut, and placenta [3]. Patients with T2DM and obesity both have lower levels of circulating omentin-1 [4]. Omentin-1 stimulates Akt phosphorylation and insulin-stimulated glucose absorption [3]. Akt pathway, a signal transduction system, increases survival and proliferation in response to extracellular inputs. Dysfunction of Akt pathway regulation, results in increased signaling activity which could lead to cancer and T2DM [5]. Additionally, omentin-1 acts via phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway to protect against arterial calcification [6]. Omentin-1 also possesses anti-inflammatory effects and inhibits TNF-α induced NF-$_k$B activation in smooth muscle cells and endothelial cells in vitro [7].

Regarding **Martin et al** which firstly demonstrated the role of glycogen synthase kinase 3 (GSK-3) in the regulation of inflammation [8]. GSK-3 α and β are serine/threonine protein kinases that are involved in the storage of glucose into glycogen. Increased GSK-3 activity is an early event in the development of insulin resistance where glycogen synthesis is impaired in type 2 diabetes (T2DM) [9]. GSK-3 was shown to support NF-κB transcriptional activity in a promoter-specific manner, demonstrating that GSK-3 selectively enhances the expression of a subset of genes activated by NF-κB [10]. GSK-3 inhibition reduces the production of pro-inflammatory cytokines [11]. Osteopontin (OPN) is a glycoprotein that is secreted into body fluids and is expressed in a number of tissues, including the kidney, endometrium, bone, and epithelium linings. OPN levels rise in chronic inflammation and play a part in arterial remodeling, and atherosclerosis [12]. OPN can influence the immune system on a variety of levels and is essential for preserving immunological homeostasis. The expression status

of OPN may be under strict control of NF- κB activation [13]. Limited studies are available about the significance of OPN levels in GDM.

According to **Winhofer et al** that have reported that the serum OPN levels were lower in GDM patients **[14]**, but the correlation between serum OPN levels and the insulin sensitivity was not statistically significant, however others found that OPN levels were similar in GDM patients and healthy pregnant **[15,16].** The regulatory mechanisms of OPN expression in GDM remain unknown. Because of the previous data, our research aims to analyze the effect of omentin-1 on OPN level and other biochemical parameters in gestational diabetic rat model and to preliminarily elucidate PI3K/AKT/GSK-3 pathway that might underlie this process.

METHODS

32 virgin female albino rats of a local strain, weighting 100–130 g, were used in this investigation, along with 6 mature males for fertilization, weighing 170–200 g. They were acquired from Zagazig University's Animal House Faculty of Veterinary Medicine. Rats were housed in the animal house at the Faculty of Medicine, Zagazig University, in steel wire cages (4/cage) that measured 50× 60× 60cm. They were provided with a standard diet, free access to water, a suitable temperature, and a regular light and dark cycle. The experimental protocol was approved by the institutional animal care and use committee of Zagazig University. The rats were divided into 4 equal groups of 8 after one week of acclimation: **Group I:** Rats in the control virgin group were given a typical chew diet consisting of 25.8% protein, 62.8% carbs, and 11.4% fat. **Group II:** Rats in the pregnant group (sham) were given normal chow throughout the trial, and on the seventh day of gestation, they received an intraperitoneal (i.p) injection using citrate buffer as a vehicle [17]. **Group III:** Rats in the GDM-induced group were fed the fatty-sucrose diet (FSD) 25% sucrose, 40% beef tallow and 20% casein protein for five weeks prior to pregnancy induction [17]. The FSD was prepared in the Department of Nutrition, Faculty of Veterinary Medicine, Zagazig University. Rats were given a single dosage of streptozotocin (i.p) on the seventh day of pregnancy (STZ) [17]. **Group IV:** The GDM+ Omentin-1 held the same protocol as group III. Rat omentin-1, (SRP8047-10 UG/vial, purchased from Sigma-Aldrich Co., Saint Louis), the dosages (100ng/kg/day), it was diluted in normal saline, chosen on the basis of prior data obtained in rats, was also administered (i.p) to the rats on the seventh day of gestation for 10 days [18**].** Rats in the other groups were given the same amount of saline using the same method.

Dead rats throughout the study were replaced to keep sample size as recommended by the animal care and use committee.

Induction of pregnancy: Females were checked for estrous cycles on two separate occasions after one week of acclimatization [19]. The male rat was placed in a separate cage with the female, who was found to be in the estrus phase (a female: male ratio of 4:1). Females were separated after mating to verify precise conception time. A vaginal smear was taken in the morning. A copulation plug or spermatozoa in the vagina served as proof of copulation. According to Klukovits et al., the presence of sperms indicated the first day of gestation [20].

Induction of experimental GDM: On the seventh day of gestation, FSD-fed rats were fasted for 16 hours before receiving a low dose of STZ (Sigma-Aldrich, U.S.A.) by (i.p) injection at a dose of 25 mg/kg dissolved in 0.1 mol/L sodium citrate (ph 4.5). The rats were then given 10% glucose solution orally six hours after the STZ injection for the following 48 hours [17]. Each rat had its blood glucose level checked using the One Touch Glucometer [21] after a sample was taken from the tail vein.

Anthropometric measures and samples collection: Rats were put in a closed plastic container, fasted overnight, and then weighed to determine the body mass index (BMI) at the start of pregnancy and on day 17 (when they were sacrificed). Using a metal ruler, we measured the length of the rat from the nose to the anus. Following that, BMI was determined using the formula body mass (g)/length (cm2); the cutoff value for an obese BMI was >0.68 g/cm2 [22]. After that, a ketamine/xylazine combination was used to anesthetize the animals. Blood samples were taken from the retro-orbital venous plexus, allowed to clot, and then centrifuged for 20 minutes at 3000 rpm in a clean plastic centrifuge tube, serum was kept at -20°C.

Tissue collection: After blood samples were taken, each mother rat of groups (II, III, and IV) was fixed in a ventro-dorsal position, and the abdomen was opened. In brief, the uteri were distinguished from the rest of the oviduct by their thick muscular walls, and then the uterus horn was torn and placentas were removed, weighed and rinsed using normal saline. At least 2 to 3 placentas were collected and snap-frozen on liquid nitrogen and stored at -70 ◦C until further processing [23]**.**

Biochemical analysis: The glucose level was evaluated using liquizyme rat Kits (GOD-PAP) in accordance with **Tietz et al** [24]**,** and the insulin

level was determined using KAP1251-INS-EASIA kits, as reported by **Temple et al.** [25] (Bio Source Europe S.A., Belgium) (Biotechnology, Egypt). With the equation HOMA-IR = fasting serum glucose (mg/dl) x fasting serum insulin (µIU/ml) /405, **Matthews et al.** [26] showed how to calculate the homeostatic model assessment of insulin resistance index (HOMA-IR). Moreover, the total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL) serum levels were measured with commercial kits as described by Tietz et al. [24] The serum low-density lipoprotein (LDL) cholesterol level was calculated using the following formula given by **Friedewald et al. [27]** : LDL (mg/dl) = (TC)[HDL+(TG/5)]. Using rat ELISA kits, the level of osteopontin (OPN) was measured in accordance with the procedure provided by **Kim et al.** [28] and the level of C reactive protein (CRP) was measured in accordance with the procedure described by **Ridker et al. [29]** (Sigma–Aldrich, Co. Cat. No: RAB0437 and RAB0097, respectively).

Measurement of Gene Expression of PI3kinase, Akt1, GSK-3, NF kappa β and osteopontin: RNA extraction; total RNA was isolated from 100 mg rat placentas using RNX™ RNA isolation kit (Sina Clon. Inc, Iran) according to the manufacturer's instructions in day 21 (after scarification of pregnant rats).The samples were treated with DNase I enzyme to avoid DNA contamination. Finally, Optical density (A260/ A280 and A260/A230) and concentration of extracted mRNA were measured. RNA samples with a ratio more than 1.8 were used for cDNA Synthesis**. [30]. cDNA synthesis:** One microgram of RNA was converted to Complementary DNA (cDNA) using the Script cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad). One microlitre of cDNA was used to perform reverse transcription (RT)-PCR using Sensimix Plus SYBR green (Alexandria,New South Wales, Australia)and primers of PI3kinase , Akt1, GSK 3, NF kappa β and osteopontin as listed below , GPDH was chosen as a suitable reference gene to normalize the m RNA expression as listed in **[Table 1]**. The PCR cycling conditions were an initial denaturation step at 95˚C for 10 minutes, followed by 40 amplification cycles of denaturation at 95˚C for 10 seconds, annealing at 60˚C and 58.8˚C for GPDH extension at 72˚C for 10 seconds. All samples were measured in duplicate. The $2^{-\Delta\Delta Ct}$ method was utilized to quantify the relative levels of gene expression **[31].**

STATISTICAL ANALYSIS

In this study's results, the mean and standard deviation were displayed (SD). The Statistical Package for the Social Sciences (SPSS), version 18, was used for the statistical analysis (SPSS Inc., Chicago, IL, United States). To compare the means of each two distinct groups, repeated measures of analysis of variance (ANOVA) were used, followed by the Student-least significant deference (LSD). Statistics were considered significant if the P value was < 0.05.

RESULTS

Effect of omentin-1 on biochemical and inflammatory parameters [Table 2]: Our results showed that the BMI of rats in group II (sham) was significantly (P<0.001) increased, while the increased serum levels of glucose, insulin, HOMA-IR, TC, TG, LDL, CRP, and osteopontin were non- significant(P>0.05), as well as the decreased serum levels of HDL (*p*>0.05) in comparison to group I(control).

However, all of these parameters significantly (p<0.001) elevated in group III (GDM) with a significant (p<0.001) reduction in the serum levels of insulin and HDL compared to other groups (I, II). Additionally, omintin-1 supplementation to rats in group IV (GDM+Omentin-1) showed a significant decline in serum levels of glucose (p<0.001), HOMA-IR (p<0.05), TC, TG, LDL, CRP, and osteopontin (p<0.001) as well as a significant (p<0.001) elevation in serum levels of HDL. While non-significant (p>0.05) changes were shown in the BMI and insulin levels compared to GDM group.

Effect of omentin-1 on PI3kinase, AKT, GSK-3, NF-κB and Osteopontin gene expression in placental tissues [Fig. 1]: As compared to pregnant group (sham), GDM showed significant (p<0.001) down-regulation of PI3 kinase and AKt, while up-regulated (p<0.001) GSK-3, NF-κB and osteopontin gene expression. On the other hand, in group IV omentin-1 treatment resulted in significant (p<0.001& p<0.01 respectively) up-regulation of PI3 kinase and AKt gene expression in concomitant with significant (p<0.001) down-regulation of GSK-3, NF-κB and osteopontin gene expression when compared to GDM and pregnant groups. The differential expression of the target gene was compared with the house keeping gene (G6PDH) in all samples.

Correlation between serum OPN and different biochemical parameters [table 3]: Our resulted overall groups reveled a significant positive correlation between serum OPN and blood glucose, TC, TG (p<0.05), HOMA-IR, CRP, GSK3 (p<0.01) and NF-kβ (p<0.001). However, no significant correlation was found between OPN and both LDL and HDL (p>0.05)

[Table 1]: Primers of PI3kinase, Akt1, GSK-3, NF kappa β and Osteopontin

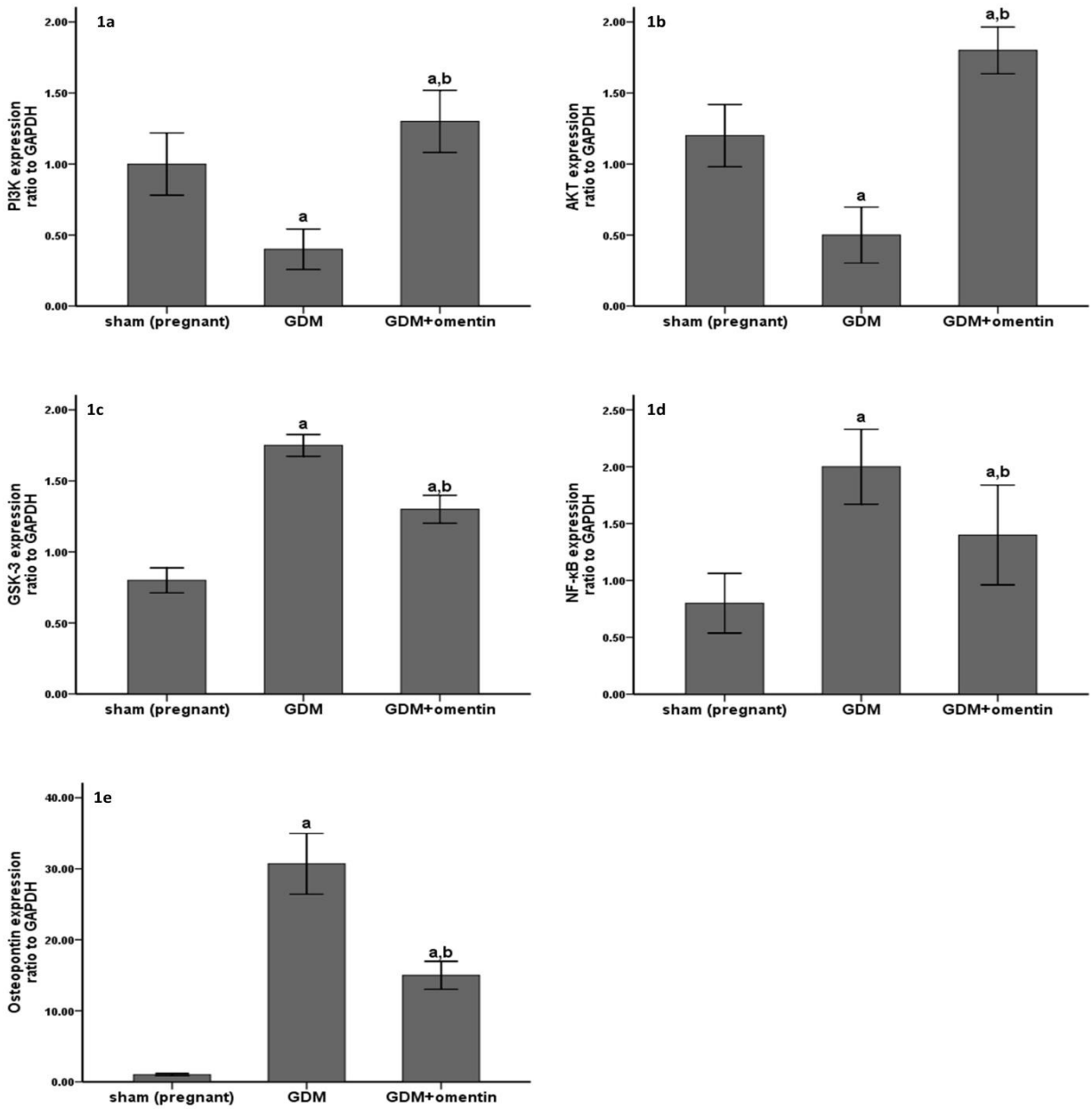
	Primers
PI3kinase	**Forward: 5'-AACACAGAAGACCAATACTC-3'** **Reverse: 5'-TTCGCCATCTACCACTAC-3'**
Akt1	**Forward: 5'-GTGGCAAGATGTGTATGAG** **Reverse: 5'-CTGGCTGAGTAGGAGAAC**
GSK 3	**Forward: 5'-GGAACTCCAACAAGGGAGCA-3',** **Reverse: 5´-TTCGGGGTCGGAAGACCTT A-3'**
NF kappa β	**Forward: 5'-CTGGTGGACACATACAGGAAGAC-3',** **reverse: 5'- ATAGGCACTGTCTTCTTTCACCTC-3'**
Osteopontin	**Forward: 5´-AGGAGAAGGCGCATTACAG-3´** **Reverse: 5´-GCTTTCATTGGAGTTGCTTG -3´**
GPDH	**Forward: 5′-TATTGGGCGCCTGGTCACCA-3′** **Reverse:5′-CCACCTTCTTGATGTCATCA-3′**

[Table 2]: Serum levels of all biochemical parameters in all studied groups

Parameters	**Group I (Control)**	**Group II (sham)**	**Group III (GDM)**	**Group IV (GDM+Omentin-1)**
BMI (g/cm2)	0.56 ± 0.03	0.68±0.05[a]	0.77±0.05[a,b]	0.75±0.06[a,b]
Glucose (mg/dL)	80.75 ± 8.2	77.06 ±7.72[a]	203.77±13.87[a,b]	165±9.18[a,b,c]
Insulin(uIU/mL)	8.62 ±1.4	9.87±2.05[a]	5.73±0.87[a,b]	5.48±1.07[a,b]
HOMA-IR	1.72 ± 0.33	1.86 ± 0.38[a]	2.9 ±0.52[a,b]	2.06 ± 0.42[a,b,c]
TC (mg/dL)	102.25±7.26	107.62±10.95	182±10.71[a,b]	144.62±11.42[a,b,c]
LDL-cholesterol (mg/dL)	52.12±5.93	55.87±6.83[a]	99.62±9.03[a,b]	71.87±8.5[a,b,a]
TG (mg/dL)	80.5 ± 7.91	82 ± 6.61[a]	165.12±11.3[a,b]	141.37±10.8[a,b,c]
HDL-C(mg/dL)	54.35 ±6.33	53.33 ±6.25[a]	28.61±5.49[a,b]	41.62 ±5.42[a,b,c]
CRP (Ug/Ml)	1.03± 0.2	1.04±0.27[a]	3.01±0.67[a,b]	1.71±0.43[a,b,c]
Osteopontien (ng/ml)	37.91± 3.06	38.85 ±3.93[a]	73.57 ±7.7[a,b]	51.41±5.38[a,b,c]

[Table 3]: Correlation between serum OPN and biochemical parameters

Osteopontin	
Parameters	**R**
Blood Glucose	0.54*
HOMA-IR	0.68**
CRP	0.63**
TC	0.51*
LDL	0.21
TG	0.54*
HDL	-0.01
GSK3	0.66**
NF-kβ	0.72***



[Figure 1]: Effect of omentin on a) PI3kinase, b) Akt, c) GSK-3, d) NF-κB and e) osteopontin gene expression. Values are expressed as mean ± SD (n = 8). Statistical analysis was done using one-way ANOVA followed by LSD test. As compared with sham (a) and GDM (b), $P < 0.05$.

DISCUSSION

Omentin-1 was frequently linked to glucose metabolism and insulin sensitivity. A decrease in omentin-1 level was found in obese, glucose intolerant and type II diabetic patients [32]. In gestational diabetic women, conflicting results were found. Some researches demonstrated a decrease in omentin-1 level in gestational diabetes [33]. Others found no significant change in omentin-1 level; however, they noticed a significant decrease in omentin-1 level all over pregnancy. Moreover, they found a decrease in its level in obese women versus normal weight control [34, 35]. These results suggest a possible role for omentin-1 in glucose and lipid metabolism during pregnancy. So, we questioned if omentin-1 administration can improve glucose homeostasis in gestational diabetic rats.

In normal pregnancy, physiologic increase in insulin resistance occurs to help adequate glucose supply to the fetus; however euglycemia is maintained by increased β-cell insulin secretion [35]. Our results in group II support these findings as we found normal blood glucose with slight increase in serum insulin and HOMA-IR but this increase did not reach statistical significance. In group III marked deterioration in insulin sensitivity (increase blood glucose and HOMA-IR) and lipid profile parameters (increase TC, TG, LDL and decrease in HDL) relative to group II were found.

Omentin-1 administration in group IV induced a significant improvement in glucose homeostatic parameters, including a significant decrease in blood glucose and HOMA-IR relative to group III. Dyslipidemia was also improved by omentin-1 treatment with a decrease in TC, TG, LDL and an increase in HDL. Similar findings were obtained by the study of **Yang et al** who demonstrated an increase in insulin-stimulated glucose transport and improvement in insulin sensitivity by in vitro omentin-1 treatment [36].

PI3K/AKT signaling pathway mediates essential physiologic functions including glucose and lipid metabolism [37]. A significant down regulation in PI3K, AKT, IRS and GLUT4 expression was detected in placentae from gestational diabetic women [38]. In our study, we found a significant decrease in placental gene expression of PI3K and AKT mRNA in group III compared to group II. While in group IV, omentin-1 treatment significantly increased PI3K and AKT mRNA expression which indicates that omentin-1 may have induced its effect on glucose and lipid metabolism using PI3K/AKT signaling pathway.

Our results are consistent with previous reports suggesting that omentin-1 perform many of its action -including its metabolic and cardiovascular protective actions- through PI3K/AKT signalling [39,36]. In addition, **Yin et al** found that omentin-1 exerted proliferative, angiogenic and antiapoptotic action on mesenchymal stem cells and this effect was probably mediated by PI3K/Akt pathway because they noticed that omentin-1 induced time and dose dependant increase in phosphorylated AKT while using LY294002 (specific inhibitor of PI3K) markedly attenuated omentin-1 induced effects [40].

GSK-3 is another molecule involved in pathogenesis of glucose intolerance and gestational diabetes. It inhibits glycogen synthase, an enzyme that stimulates glycogen synthesis to store glucose. An increase in GSK3 activity was associated with insulin resistance and impairment of glycogen synthesis in diabetic patients [41]. Activation of PI3K/AKT pathway was found to phosphorylate and hence inhibits GSK3 [42] which is consistent with our results as we found a significant increase in GSK3 gene expression in group III compared to group II concomitant with the decrease in PI3K and AKT expression, while omentin-1 treatment in group IV, significantly decreased GSK3 expression. Matching with this, an increase in GSK3 mRNA expression [43] and activity [44] was reported in adipose tissue from women with GDM. **Yin et al** found that treatment of mesenchymal stem cells with omentin-1 caused phosphorylation and inactivation of GSK3 through PI3K/AKT pathway as inhibition of PI3K by LY294002 blocked this effect [40]. Taken together, omentin-1 improved glucose metabolism by activation of PI3K/AKT signaling pathway and inhibition of GSK3 leading to improvement of glucose tolerance and decrease in insulin resistance

The anti-inflammatory effect of omentin-1 could play a role in modulation of insulin sensitivity. In this study, we found a decrease in C reactive protein in group IV after omentin-1 treatment compared with group III. Omentin-1 treatment was previously described to decrease TNF-α and CRP [45]. Moreover, in endothelial cell culture, omentin-1 was able to suppress inflammatory response [46]. The anti-inflammatory effect of omentin-1 can also be indirect through its effect on lipid metabolism as activation of PI3K/AKT pathway by omentin-1, inhibits lipolysis and stimulates lipid biosynthesis by affecting sterol regulatory element-binding proteins (SREBP) and FOXO1. SREBP controls fatty acid synthase and also cholesterol related genes and FOXO1 decreases adipose triglyceride lipase [47,48]. So omentin-1 can improve dyslipidemia and decrease obesity mediated low grade inflammatory response which can induce and exacerbate insulin resistance.

Osteopontin (OPN) plays a role in many physiologic and pathologic conditions as tissue remodeling and bio-mineralization [12]. It was described as a pro-inflammatory cytokine that share in immune modulation as it helps monocytes/macrophages recruitment and mediates cytokine secretion by leukocytes [49]. OPN is particularly involved in chronic inflammatory disorders and is believed to be involved in adipose tissue inflammation and insulin resistance [50]. In our study, we found a significant increase in serum OPN level in group III compared to normal pregnant group and these levels showed significant decrease in omentin-1 treated group. Placental OPN gene expression in gestational diabetic rats was also markedly elevated (multiple folds) and the increase was highly significant relative to group II and it decreased significantly in group IV by omentin-1 administration. NF-κB was reported to be implicated in OPN transcription and its binding site was identified on OPN gene [13]. We found a significant increase in NF-κB gene expression in gestational diabetic group versus group II, while its expression significantly decreased with omentin-1 treatment in group IV and these changes positively correlated with OPN level. Matching with our results, NF-κB was previously reported to be inhibited by omentin-1 as treatment of human umbilical vein endothelial cells with omentin-1 inhibited TNF-α mediated signaling pathway of NF-κB through inhibiting the degradation of NF-κB inhibitory protein (IκBα) and decreasing NF-

κB/DNA binding activity [51]. On the other hand, GSK3 was found to enhance NF-κB transcriptional activity [10], while mice fibroblasts of GSK3 deficient embryos showed reduced NF-κB function [52]. These results indicate that the favorable effects of omentin-1 might be partially mediated through down-regulation of OPN expression probably by inhibiting NF-κB either by a direct effect of omentin-1 or indirectly by decreasing GSK3 expression as shown in our results.

Similar to our study, **Housseiny et al** found significant OPN overexpression in the endometrium of diabetic rats during the implantation widow when compared with the control group [53]. Conversely, in human, **Saklamaz et al** and **Saucedo et al** found no significant change in serum OPN in gestational diabetic women [15, 16]; others reported lower OPN level in women with GDM [14], these discrepancies may be related to species difference or different timing or procedure of assay.

CONCLUSION

Omentin-1 administration in gestational diabetic rats has favorable effect on glucose and lipid metabolism leading to improvement of insulin sensitivity and attenuation of inflammatory response through PI3K/AKT/GSK3 pathway and through down-regulation of osteopontin expression suggesting the possible use of omentin-1 in treatment of glucose intolerance to decrease the adverse maternal and fetal outcomes of gestational diabetes. Therapeutic targeting of PI3K/AKT pathway or GSK-3 inhibitors can be promising points for future research.

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