Expression of Adiponectin Receptors in Human Placenta and Its Possible Implication in Gestational Diabetes

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Abstract: Problem statement: Similar to obese patients and type 2 diabetic patients, adiponectin levels are reduced in former Gestational Diabetes Mellitus (GDM) patients and are lower in GDM women during late pregnancy compared with pregnant control subjects matched for BMI. Diabetic insult at later stages in gestation, such as may occur in gestational diabetes, will foremost lead to short-term changes in a variety of molecules for key functions including gene expression in the placenta. **Approach:** In this study we assessed the expression of adiponectin receptors in human placenta to identify the site (s) of expression and to clarify the effect of gestational diabetes in this expression. This study was carried on 10 normoglycemic pregnant women and 20 GDM women. The placental tissue was collected immediately after delivery and tissue biopsies were taken from both fetal and maternal sides of each placenta. One step-RT-PCR for ADIPOR1 and ADIPOR2 was done by Real Time PCR using Syber Green technique. Relative quantification of mRNA of the ADIPOR1 and ADIPOR2 genes was measured using ABI7900 Real Time machine. **Results:** Both types of Adiponectin Receptors (ADIPOR1 and ADIPOR2) are expressed in human placenta. ADIPOR1 is more highly expressed than ADIPOR2 in both fetal and maternal sides of GDM cases and normal pregnant women. ADIPOR1 mRNA expression was significantly up regulated in GDM women compared to normal pregnant women, whereas no significant difference in the expression of ADIPOR2 was detected between the two groups. There was no evidence of maternal-fetal side difference in the expression of adiponectin receptors in GDM cases but in normal pregnant women there is a statistically significant difference between both sides in the expression of both ADIPOR1 and ADIPOR2. **Conclusion:** We concluded that adiponectin plays an important role in mediation the glucose metabolism in fetal tissues through its receptors, mainly Adiponectin Receptor 1 (ADIPOR1).

Key words: Adiponectin, gestational diabetes, gene expression, real time PCR, placenta receptors

INTRODUCTION

Adiponectin is a novel adipose tissue-specific protein abundantly expressed in human and rodent fat and secreted from differentiated adipocytes (Combs *et al*., 2004). Adiponectin is present at high concentrations in the circulation as a low molecular weight complex consisting of a dimer of trimers as well as a high molecular weight complex of up to six trimers (Pajvani *et al*., 2003). Circulating levels of adiponectin are inversely related to the degree of adiposity and are positively associated with insulin sensitivity in both healthy subjects and diabetic patients. Plasma adiponectin levels decreased in parallel to the progression of insulin resistance, suggesting that a reduction in circulating adiponectin may be related to the development of insulin resistance (Haluzik *et al*., 2004). Two Adiponectin Receptors (ADIPOR1 and ADIPOR2) have recently been identified. ADIPOR1 is abundantly expressed in muscle whereas ADIPOR2 is predominantly expressed in liver (Yamauchi *et al*., 2003). Activation of these receptors phosphorylates AMP-Activated Protein Kinase (AMPK), a regulator of energy homeostasis of the cell and stimulates fatty acid oxidation and glucose uptake (Yamauchi *et al*., 2007).

Placental function is regulated at least in part by a wide spectrum of cytokines that are produced both locally and distally and are essential for adapting the maternal metabolism to pregnancy to assure normal placental development and fetal growth (Caminos *et al*.,

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2005). The human placenta expresses virtually all known cytokines including tumor necrosis factor TNF-α, resistin and leptin, which are also produced by the adipose cells. The discovery that some of these adipokines especially adiponectin are key players in regulation of insulin action suggests possible novel interactions between the placenta and adipose tissue in understanding pregnancy induced insulin resistance. The interplay between the two systems becomes more evident in Gestational Diabetes Mellitus (GDM) (Desoye and Hauguel, 2007). Also previous studies demonstrated that lower concentrations of adiponectin have been consistently reported in patients with gestational diabetes as compared to patients with normal pregnancy (Ategbo *et al.*, 2006) Moreover, pregnant patients with low concentrations of adiponectin during the first trimester are more likely to develop gestational diabetes compared to those with normal concentrations of this hormone (Williams *et al.*, 2004).

The present study was undertaken to assess the expression of adiponectin receptors in human placenta, identify the site (s) of expression and clarify the effect of gestational diabetes in that expression.

MATERIALS AND METHODS

Pregnant women attending the obstetric and gynecology department, Benha University Hospital for antenatal care between April and June 2009 were consecutively recruited for participation in this study. The procedures used in this study followed the protocol approved by the local institutional review board. The research objectives were explained to each patient and consent was obtained. The study was carried on thirty pregnant women designed as control group, which included ten normoglycemic pregnant women and had not any pregnancy disorders and patient group, which included twenty gestational diabetic women who had the gestational diabetes as the only pregnancy disorder. All of the patients were diagnosed as gestational diabetic women by having a fasting plasma glucose level >126 mg Dl^{-1} or a random plasma glucose level >200 mg Dl^{-1} that is confirmed on a subsequent day (American Diabetes Association, 2009). All women were followed up until delivery and all relevant clinical information were recorded to exclude cases with any other pregnancy disorder. Hospital patient records were later examined for patient age, height, pre-pregnancy weight, infant birth weight, medication, age of gestation, mode of delivery, medical condition and recorded for analysis. Body Mass Index (BMI) is calculated. All the twenty gestational diabetic women were taking insulin therapy.

Tissue handling: About 1cm³ of placental tissue was collected immediately after delivery and tissue biopsies were taken from both fetal and maternal sides, placed in cry tubes and stored in RNA later solution (RNA stabilizing reagent) (Qiagen Inc., Valencia, CA) at 10 µL per 1 mg of tissue then stored at -80°C for further processing.

Total RNA extraction: From each of the stored biopsies, 30 mg of tissue were cut and weighed before complete throwing. The weighed samples were homogenized using tissue ruptor homogenizer (Qiagen, Inc.,) for 20 sec. Total RNA was extracted using RNeasy mini kit (Qiagen. Inc.,) following the standard protocol. 50 µL of the eluted RNA was collected immediately, placed in ice or stored at -20°C. A260 and A280 were taken by UV spectrophotometer (Optima SP-3000+, Japan). Pure RNA has an A260/A280 ratio of 1.9-2.3.

Relative quantization of mRNA of the respective genes by real time PCR using SYBR GREEN: In this study, we did one-step RT-PCR using QuantiTect® SYBR® Green RT-PCR master mix kit (Qiagen. GmbH). In ABI 7900 (Applied Biosystem, Real time thermal cycler) the prepared reaction components were done in 96 well PCR plate Using Real time cycler conditions of 50°C, 30 min, (Reverse transcription) -95°C, 15 min, (Initial denaturation) followed by 40 cycles of 94°C, 30 sec, 55°C, 1 min and 72°C, 1 min for Denaturation, Annealing, Extension steps respectively. Primer sequence of human ADIPOR1 was 5'-AAACTGGCAACATCTGGACC-3' (5'-3' sequence forward) and 5′-GCTGTGGGGAGCAGT AGAAG-3' (5'-3' sequence reverse). Primer sequence of human adiponectin receptor ADIPOR2 5'-ACAGGCAACATTTGG ACACA-3' (5'-3' sequence forward) and 5'-CCAAGGAACAAAACTTCCCA-3′ (5'-3' sequence reverse). Primer sequence of Glyceraldehyde Phosphate Dehydrogenase (GAPDH) as internal control (housekeeping gene) was 5' - TGATGACATCAAGAAGGTGGTGAAG-3'(5'-3' sequence forward) and 5'-TCCTTGGAGGCCATGTGGGCCAT-3' (5'-3' sequence reverse). The PCR primers were synthesized by (Operon, inc., Huntsville, Alabama Germany). According to the RQ manager program ABI SDS software (ABI 7900), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale). The Threshold Cycle (CT) serves as a tool for

calculation of the starting template amount in each sample. Fold expression changes are calculated using the equation 2-ΔΔct.

Statistical analyses: The results were presented as means ± SD, Comparisons of categorical variables were made between cases and controls using chi-squared exact test. The Spearman's correlation coefficient was used to measure the closeness of a linear relationship between relative gene expression values derived from tissue sampled from maternal and fetal sides of the placenta. All analysis was performed using the Statistics Package St. Social Sciences (SPSS) and Microsoft office Excel is used for data processing and data analysis. Differences are considered as statistically significant for a p value less than 0.05.

RESULTS

The maternal clinical characteristics of GDM and normal pregnant women are listed in Table 1.

Both types of receptors (ADIPOR1 and ADIPOR2) are expressed in human placenta in both fetal and maternal sides Fig. 1. ADIPOR1 is more highly expressed than ADIPOR2 in both placental sides of GDM cases and normal pregnant women. Mean value of relative ADIPOR1 and ADIPOR2 expression in the fetal side of GDM cases was 226.8±76.3 and 19.1±4.9 while that of normal pregnant women was 53.2±20.6 and 16.9±1.9 respectively. Also it was of maternal side of GDM cases 228.80±73.6 and 19.1±4.9 while that of normal pregnant women was 57.8±18.9 and 18.6±2.3 respectively. ADIPOR1 gene expression was 4.3 folds higher in fetal side of placenta of GDM cases relative to normal pregnant women (p<0.001) and in the maternal side it was 3.96 folds higher in GDM cases relative to normal pregnant women (p<0.001), whereas no significant difference in the expression of ADIPOR2 was detected between the two groups (p>0.05) as ADIPOR2 gene expression was only 1.13 fold higher in fetal side of placenta of GDM cases relative to normal pregnant women (p>0.05) and in the maternal side it was only 1.03 fold higher in GDM cases relative to normal pregnant women (p>0.05) Fig. 1.

There was a statistically significant difference between maternal and fetal sides in the expression of both ADIPOR1 and ADIPOR2 in normal pregnant women (p values<0.05), while no evidence of a significant difference between both sides in GDM cases (p>0.05).

A positive correlation (although not reaching a statistically significant value) was detected between

BMI and the expression of ADIPOR1 in normal pregnant women. But in GDM women, the correlation was negative (also not reaching a statistically significant level) p>0.05 Fig. 2 and 3.
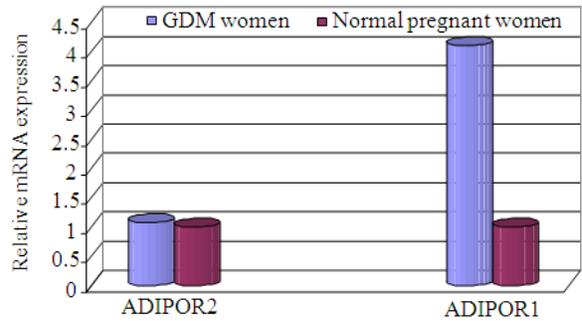

Fig. 1: Fold changes of ADIPORs relative gene expression levels in placenta from women with gestational diabetes mellitus) relative to normal pregnant women
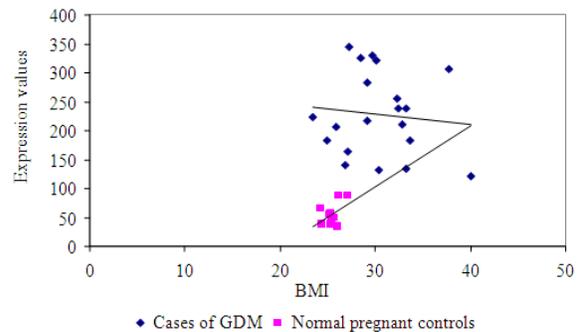

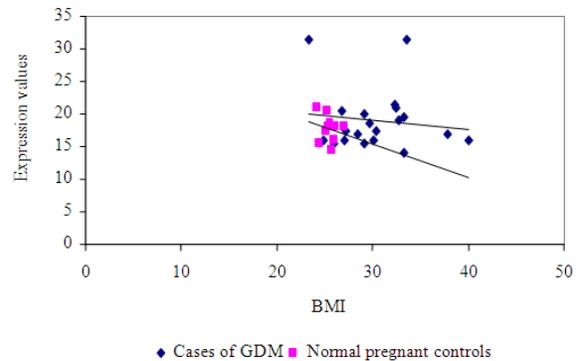Fig. 2: Correlation coefficient (r) between BMI and the expression of ADIPOR1

Fig. 3: Correlation coefficient (r) between BMI and the expression of ADIPOR2

Table 1: Mean values ± SD of some clinical characteristics of the studied groups

Parameters	Groups		Student "t"	p	95%CI
	GDM cases n = 20	Normal pregnant controls n = 10			
Maternal age	29.9±4.8800	25.00±2.8300	2.900	<0.01*	1.5-8.300
Pre pregnancy weight	80.3±10.970	68.30±7.290	3.120	<0.01*	4.1-19.90
BMI	30.4±4.1600	25.50±0.830	3.660	<0.01*	2.15-7.64
Gestational age at delivery	38.33±0.680	38.38±1.28	0.127	>0.05	-0.77-0.68
Infant birth weight	4.39±0.370	2.85±0.37	10.700	<0.001*	1.2-18.00

$p<0.01$*: Significant; $p>0.05$: Non significant; GDM: Gestational Diabetes Mellitus; BMI: Body Mass Index n: number

DISCUSSION

The hypothesis that adiponectin may play a role in normal and complicated pregnancies is based on several findings of previous studies which showed that one of the hallmarks of human pregnancy is insulin resistance (Catalano *et al*., 2006).

The statistically significant increases in the maternal age, pre-pregnancy weight, BMI and birth weight of GDM women are in agreement with those published by American Diabetes Association (2009) which considered that a maternal age >25 years, increased weight and BMI before pregnancy are risk factors for GDM. Both types of receptors (ADIPOR1 and ADIPOR2) are present in human placenta in both fetal and maternal sides of GDM cases and normal pregnant women and these findings were in agreement with those of Meller *et al*. (2006); Chen *et al*. (2006) and Kleiblova *et al*. (2010) while Caminos *et al*. (2005) demonstrated the presence of only ADIPOR2 in human and rat placenta. ADIPOR1 is more highly expressed than ADIPOR2 in both fetal and maternal sides of GDM cases and normal pregnant women. These findings were in agreement with those of Meller *et al*. (2006). The increase in adiponectin receptors gene expression could be due to the decreased adiponectin concentrations in GDM cases (Ategbo *et al*., 2006) and generally consistent with the published study of Chen *et al*. (2006) that showed a significant increase in ADIPOR1 mRNA in GDM placenta (90% increase compared with normal), whereas no significant up-regulation in the expression of ADIPOR2 was detected between the two groups. These results differ with those of Meller *et al*. (2006) and Kleiblova *et al*. (2010) which showed that there was no significant changes were seen in GDM cases compared with controls in ADIPOR1 gene expression. Severity of gestational diabetes mellitus as well as the method of glycolic control after GDM diagnosis may account for the disparity seen between these studies.

Both maternal and fetal sides of the placenta were separately studied to see the influence on gene expression due to site of selection. It was found that there was no evidence of maternal-fetal side difference in the expression of adiponectin receptors in GDM cases and this is consistent with published studies by Meller *et al*. (2006) who also found that there was no evidence of maternal-fetal side difference in the expression of adiponectin receptors. While the statistically significant difference that we found between maternal and fetal sides in the expression of both ADIPOR1 and ADIPOR2 in normal pregnant women. We think that we should have taken a larger number of samples when looking for significant differences in gene expression as previous studies revealed that the amounts of transcripts differ considerably within and between each placenta (Pidoux *et al*., 2004).

A limitation of this study is the use of biopsies to represent maternal and fetal tissues in term placentas. During pregnancy cytotrophoblast cells, which invade from the fetal tissue, form anchoring villi and anchor into the maternal decidua basalis (Junqueira and Carneiro, 2003). Therefore, samples taken from the maternal side in this study, although consisting physically of some maternal cells, are not exclusively comprised of these cell types.

There is a positive correlation (although not reaching a statistically significant value) between BMI and the expression of ADIPOR1 in normal pregnant women. Overweight pregnant women had significantly lower adiponectin concentration than normal weight women (Nien *et al*., 2007) which explains the increase in the receptors expression associated with increased BMI. In GDM women our results showed a negative correlation (although not reaching a statistically significant level) between BMI and the expression of ADIPOR1 which may be explained by the effect of insulin therapy taken by these group of patients and this is in agreement with Tsuchida *et al*. (2004) who demonstrated that adiponectin receptors are negatively regulated by insulin.

CONCLUSION

ADIPOR1 is more highly expressed than ADIPOR2 in human placenta; ADIPOR1 is significantly upregulated in GDM women. Thus, adiponectin plays an important role in mediation the glucose metabolism in fetal tissues through its

receptors, mainly Adiponectin Receptor 1 (ADIPOR1). So, one of the therapeutic strategy in GDM may include the use of small molecule agonists to ADIPOR1 mainly in order to improve glucose metabolism in the fetus and decrease fetal macrosomia and morbidity. Future studies should include micro-dissection of cells from maternal and fetal origin for separate analysis to show conclusively whether there is any correlation in gene expression between these two compartments .Future studies should have a larger number of samples when looking for significant differences in gene expression as previous studies revealed that the amounts of transcripts differ considerably within and between each placenta.

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