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RESEARCH ARTICLE

RECIPROCAL RELATIONSHIP BETWEEN INSULIN RESISTANCE AND DISTURBED ADMA/NO AXIS IN GESTATIONAL DIABETES MELLITUS.

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Abstract

Objectives: To estimate serum nitric oxide (NO) and asymmetric dimethylarginine (ADMA) levels in pregnant women and correlate it with severity of gestational diabetes mellitus (GDM) judged by Oral Glucose Tolerance Test (OGTT).

Patients & Methods: 225 pregnant women gave fasting blood samples to estimate baseline blood glucose (BG), and serum insulin, NO and ADMA, and to undergo OGTT. Insulin resistance (IR) was indicated by HOMA-IR score >2. At 24th week, blood sample were re-evaluated using OGTT and women were categorized in Study (GDM) and Control (free of GDM) group.

Results: At 24th week, HOMA-IR scores were significantly higher than baseline scores and in study versus control women. Baseline serum ADMA levels were significantly higher, while serum NO levels were significantly lower in study versus control women and correlated with body mass index (BMI), GDM, BG and HOMA-IR score of all women. Serum ADMA and NO showed significant correlation with extent of serum insulin change, but low serum NO is significant predictor for development and severity of IR.

Conclusion: IR induces endothelial dysfunction manifested as low serum NO and high serum ADMA. Baseline serum NO could predict the extent of serum insulin change.

Introduction:-

Insulin resistance (IR) is defined clinically as the inability of a known quantity of insulin to increase glucose uptake and utilization in an individual as much as it does in normal one [(1)]. IR is a key feature of obesity, metabolic syndrome and type-2 diabetes mellitus (T2DM) [(2)] and results in several metabolic and vascular phenomena that promote the development of atherosclerosis, hypertension and accelerated development of coronary heart diseases [(3)].

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Diabetes mellitus (DM) complicates 3-10% of pregnancies, resulting in significant maternal and neonatal morbidity and mortality [4]. Women developed gestational (GDM) diabetes share subjects predisposed to T2DM similar characteristics as IR which aggravates the condition [5].

The mechanisms underlying either normal or abnormal antepartum β-cell adaptation in GDM are unclear [6]. However, high maternal blood glucose levels induces adverse intrauterine environment, which has a negative impact on health outcomes for both the mother and the offspring [7].

GDM is a health problem with deleterious effects on maternal health not limited by delivery but may prolong and expose these women to long-term medical problems [8] as women with GDM are at increased risk of developing manifest T2-DM [9. 10] and had a significantly increased incidence of hypertension and ischemic heart diseases [10].

Nitric oxide is synthesized from L-Arginine by three nitric oxide synthase (NOS) isoforms and its synthesis is selectively inhibited by asymmetric dimethylarginine (ADMA), which results from cellular protein degradation [11]. ADMA as a competitive endogenous inhibitor of NOS, thus elevated ADMA levels may play a key role in the pathophysiology of endothelial dysfunction, and in the progression of atherosclerosis and cardiovascular diseases [12]. NO synthesis and ADMA generation occurs intracellularly, however, these biomarkers are usually measured in plasma [14].

Hypothesis:-
The authors previously reported that disturbed maternal serum ADMA/NO axis and low E_2 serum levels may underlie pre-eclampsia development [14]; so as another trial the current study supposed a similar relation between disturbed maternal serum ADMA/NO axis and development of GDM in non-diabetic pregnant women who were free of history of GDM.

Design:-
Prospective comparative clinical trial

Setting:-
Benha & Tanta Universities and Insurance Hospitals

Aim of work:-
This study aimed to estimate serum NO and ADMA in pregnant women and to correlate these levels with frequency and severity of GDM as judged by the results of the 75g Oral Glucose Tolerance Test (75-OGTT).

Patients & Methods:-

The study protocol was approved by the Local Ethical Committee and only women signed a written fully informed consent were included in the study. All pregnant women who attended the Antenatal Outpatient Clinics (OPC), at Benha and Tanta University and Insurance hospitals for assurance of diagnosis of being pregnant were eligible for study inclusion. At 6th week gestational age (GA), women's demographic data including age, weight and height were determined for calculation of body mass index (BMI) in kg/m^2 as weight (kg)/ height (m^2) and patients were classified according to BMI using the World Health Organization ranges [15].

Baseline clinical and obstetric data were collected and verified to assure inclusion and exclusion criteria which include pre-pregnancy DM, history of previous GDM, BMI>35 kg/m^2 and multiple pregnancy. Then, all patients were referred to General Medicine clinic for exclusion of essential hypertension, vasculitis, renal or hepatic diseases. Women lost during the course of pregnancy were excluded.

At time of the 1st antenatal visit, all women underwent 50-gm glucose challenge test (50-GCT) where 50 gm of glucose were orally taken, irrespective of fasting state of women, and after 1-hr a venous blood sample was obtained for estimation of blood glucose and at cutoff point of 140 mg/ml indicates a positive 50-GCT [16]. All women were asked to attend the OPC one week later while overnight fasting to give blood samples to determine baseline fasting blood glucose (FBG), and serum levels of insulin, NO and ADMA, and to undergo the 75-OGTT. The 75-OGTT entails obtaining three blood samples; a fasting sample and two samples 1-hr and 2-hrs after taking an oral snake containing 75 gm glucose for estimation of postprandial blood glucose (PPBG)

Baseline insulin resistance (IR) was evaluated using the homeostasis model assessment of IR (HOMA-IR) on the basis of insulin and glucose levels and according to the formula fasting serum insulin (µU/ml) x [fasting plasma glucose (mg/ml)/18])/22.5; HOMA-IR score of >2 is considered abnormal [17]. Then, all women were asked to attend overnight fasting to the OPC at the 24th week GA to give blood sample for re-estimation of serum insulin and undergo the 75-OGTT and HOMA-IR score was re-calculated.

The study plan was to include pregnant women who developed GDM at the 24th week visit as study group and women who were free of GDM till the 24th week GA as control group. GDM was defined according to the

American Diabetes Association [18] as the first recognition of any degree of glucose intolerance during pregnancy and determined according to the results of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study [19] and International association of diabetes and pregnancy study groups (IADPSG) recommendations [20] as follows: FBG ≥92 mg/dl, 1-h BG ≥180 mg/dl and 2-h BG ≥153 mg/dl.

Investigations:-

Sampling:- Venous blood samples (5 ml) were collected from the antecubital vein under complete aseptic conditions and were divided into two parts:

1. The first part was put in a tube containing sodium fluoride (2 mg sodium fluoride/ ml blood) to prevent glycolysis and plasma was separated by centrifugation to be used for estimation of blood glucose levels.
2. The second part was collected in plain tube, allowed to clot, centrifuged at 1500×g for 15 min and the serum samples were collected in clean dry Eppindorff tube to be stored at –70°C until assayed.

Estimated parameters:-

1. Blood glucose levels were estimated using glucose oxidase method [21].
2. Serum insulin levels were determined using ELYSA kit (Enzymuntest Insulin, ES 600, Boehringer Mannheim) [22].
3. Colorimetric estimation of serum NO levels were [23].
4. Serum ADMA levels were measured using ELISA kit (Eagle Biosciences, Hamburg, Germany) [24].

Statistical analysis:-

Obtained data were presented as mean±SD, numbers and percentages. Results were analyzed using One-way ANOVA with post-hoc Tukey HSD Test and Chi-square test (X^2 test). Possible relationships were investigated using Pearson's linear regression. Sensitivity & specificity of estimated parameters as predictors were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that AUC=0.05. Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) for Windows statistical package. P value <0.05 was considered statistically significant.

Results:-

Throughout the duration of the study, 253 women were eligible for evaluation, 28 women were excluded and 225 women were included in the study (Fig. 1). At time of enrolment, the 50-GCT detected 94 positive women and 131 negative women, while during follow-up the 75-OGTT defined 29 women (12.9%) as having GDM (Study group), while 196 women had normal 75-OGTT (Control group). Seventeen women of those gave positive 50-GCT developed GDM, while 77 women completed their pregnancy course free of GDM and despite of the significantly (p=0.048) higher frequency of women had positive 50-GCT among study versus control women, 50-GCT showed sensitivity and specificity rates of 58.2% and 60.7%, respectively and accuracy rate of 60.4% for prediction of development of GDM. Revision of demographic and clinical enrolment data showed non-significant (p>0.05) difference between women of both groups apart from body weight and BMI that were significantly (p=0.013 & 0.023, respectively) higher in study versus control women (Table 1, Fig. 1).

Baseline mean levels of FBG, serum insulin and calculated HOMA-IR scores were non-significantly (p>0.05) higher in study versus control women, while at the 24th week GA, all women showed significantly (p<0.05) higher levels of FBG and PPBG, serum insulin and HOMA-IR scores compared to baseline levels with significantly (p<0.05) higher levels in study versus control women. Moreover, baseline serum ADMA levels were significantly (p<0.05) higher, while serum NO levels were significantly (p<0.05) lower in study versus control women (Table 2).

Baseline serum ADMA and NO levels showed significant correlation with development of GDM. Interestingly, serum ADMA and NO showed significant correlation with HOMA-IR index and its components; blood glucose and serum insulin levels, in both controls and study groups. Moreover, estimated serum ADMA and NO levels showed significant correlation with baseline body weight and BMI. Such correlations were positive with ADMA and negative with NO, but the correlations were pronounced in study versus control women (Table 3).

Analysis of baseline data and laboratory findings using ROC curve analysis defined low baseline serum NO, high BW, high serum ADMA and high BMI as significant predictors for development of GDM, in decreasing order of significance (Table 4). Also, ROC curve analysis defined high baseline serum ADMA as a specific and low baseline NO as sensitive predictor the development of GDM (Fig. 2).

Baseline serum ADMA and NO showed significant correlation with the extent of change in serum insulin since 1-wk after enrolment till 24th wk GA with the correlation being positive with ADMA (r=0.202, p=0.002) and negative with NO (r=-0.301, p=0.0005). However, ROC curve analysis defined low serum NO as a significant predictor for possibility of development of IR manifested as high blood glucose level despite of the high serum insulin level (Fig. 3).

Table 1:- Patients' data determined at time of enrolment

Data		Control group (n=196)	Study group (n=29)	P value
Age (years)		28.3±2.5	27.6±2.6	0.155
Weight (kg)		81.4±8.4	85.6±7.6	0.013*
Height (cm)		169.6±3.4	169.9±3.1	0.186
BMI (kg/m^2)		28.3±3	29.6±2.7	0.023*
Gravidity		2±0.8	1.7±0.7	0.091
Parity		0.9±0.8	0.7±0.7	0.206
Blood pressure	Systolic	113±7.7	112.5±5.9	0.685
	Diastolic	72.9±4.9	74±4.1	0.478
Fasting blood glucose (mg/dl)		83.2±10.1	86.4±12.5	0.124
50-GCT	Positive	77 (39.3%)	17 (58.6%)	0.048*
	Negative	119 (60.7%)	12 (41.4%)	

Data are presented as mean±SD, numbers & percentages; BMI: Body mass index; 50-GCT: 50 gm Glucose challenge test; *: indicates significant difference

Table 2:-Patients' laboratory data determined at 1-wk after enrolment and at 24th week GA with calculated HOMA-IR score

			Control group (n=196)	Study group (n=29)	P value
Baseline (1-wk after enrollment)	75-OGTT	Fasting level	84.7±11.5	88.6±15.8	0.105
		1-hr PP level	127.4±12.8	132±16.5	0.082
		2-hr PP level	95.2±8.6	102.9±10.2	0.071
	Serum insulin (µU/ml)		8.1±2.5	8.7±2.2	0.261
	HOMA-IR score	<2	132 (67.3%)	16 (55.2%)	0.197
		>2	64 (32.7%)	13 (44.8%)	
		Mean level	1.73±0.63	1.93±0.58	0.118
	Serum ADMA (ng/ml)		3.11±1.36	4.29±1.8	0.001*
	NO (µmol/L)		40.21±1.8	29±4.6	0.001*
At 24th wk GA	75-OGTT	Fasting level	97.5±5	116.7±7.1	0.001*
		1-hr PP level	159.3±10.1	216.3±16.2	0.001*
		2-hr PP level	122.4±5.7	162.1±11.6	0.001*
	Serum insulin (µU/ml)		10.4±5.8	16.8±3.4	0.001*
	HOMA-IR index	<2	130 (60.7%)	0	0.001*
		>2	66 (39.3%)	29 (100%)	
		P1=	0.831	0.001*	
		Mean level	2.6±1.5	4.9±1	=0.001*

		P1=	0.001*	0.001*	

Data are presented as mean±SD & numbers; percentages are in parenthesis; GDM: Gestational diabetes mellitus; 75-OGTT: 75-gm oral glucose tolerance test; GA: Gestational age; PP: Postprandial; HOMA-IR score: Homeostasis model assessment of insulin resistance; *: indicates significant difference

Table 3:- Correlation coefficient between baseline serum levels of ADMA and NO and development of GDM, demographic data and IR parameters

Serum parameter		ADMA						NO					
Group		Control		Study		Total		Control		Study		Total	
Variable		r	p	r	p	r	p	r	p	r	p	r	p
GDM						0.268	<0.001					-0.519	<0.001
Age		0.191	>0.05	0.113	>0.05	0.115	>0.05	0.165	>0.05	-0.123	>0.05	0.241	>0.05
Weight (kg)		0.222	>0.05	0.401	<0.001	0.397	<0.001	-0.440	0.017	0.132	>0.05	-0.109	>0.05
BMI (kg/m^2)		0.364	0.049	0.393	<0.001	0.383	<0.001	-0.220	>0.05	0.141	>0.05	-0.159	>0.05
GCT		0.274	0.05	0.196	>0.05	0.223	0.001	-0.166	>0.05	-0.128	>0.05	-0.222	0.001
Baseline	BG	0.510	0.005	0.154	>0.05	0.134	>0.05	-0.145	>0.05	-0.096	>0.05	-0.116	>0.05
	SI	0.317	>0.05	0.701	<0.001	0.634	<0.001	-0.161	>0.05	-0.172	0.016	-0.178	0.007
	HOMA	0.414	0.026	0.601	<0.001	0.570	<0.001	-0.159	>0.05	-0.179	0.015	-0.192	0.004
20th w	BG	0.451	0.014	0.316	<0.001	0.404	<0.001	-0.208	>0.05	-0.163	>0.05	-0.465	<0.001
	PI	0.321	>0.05	0.245	0.001	0.317	<0.001	-0.534	0.003	-0.204	0.004	-0.346	<0.001
	HOMA	0.427	0.021	0.425	<0.001	0.842	<0.001	-0.453	0.019	-0.142	>0.05	-0.318	<0.001

GDM: Gestational diabetes mellitus; BMI: Body mass index; GCT: Glucose challenge test; BG: Blood glucose; SI: Serum insulin; HOMA-IR: Homeostasis model assessment of insulin resistance; ADMA: asymmetric dimethylarginine; NO: Nitric oxide

Table 4:- ROC curve analysis of baseline patients' data and laboratory findings as predictors for development of GDM

	AUC	P value	95%CI
Age	0.425	0.193	0.306-0.544
Weight (kg)	0.678	0.002	0.571-0.786
BMI (kg/m^2)	0.660	0.005	0.553-0.766

Fasting blood glucose (mg/dl)	0.518	0.755	0.411-0.625
Fasting serum insulin (µU/ml)	0.565	0.258	0.459-0.671
HOMA-IR	0.574	0.200	0.467-0.680
Serum ADMA (ng/ml)	0.668	0.003	0.557-0.780
Serum NO (µmol/L)	0.078	0.0003	0.033-0.123

AUC:- Area under curve; 95%CI: 95% confidence interval; GDM: Gestational diabetes mellitus; BMI: Body mass index; ADMA: Asymmetric Dimethylarginine; NO: Nitric oxide

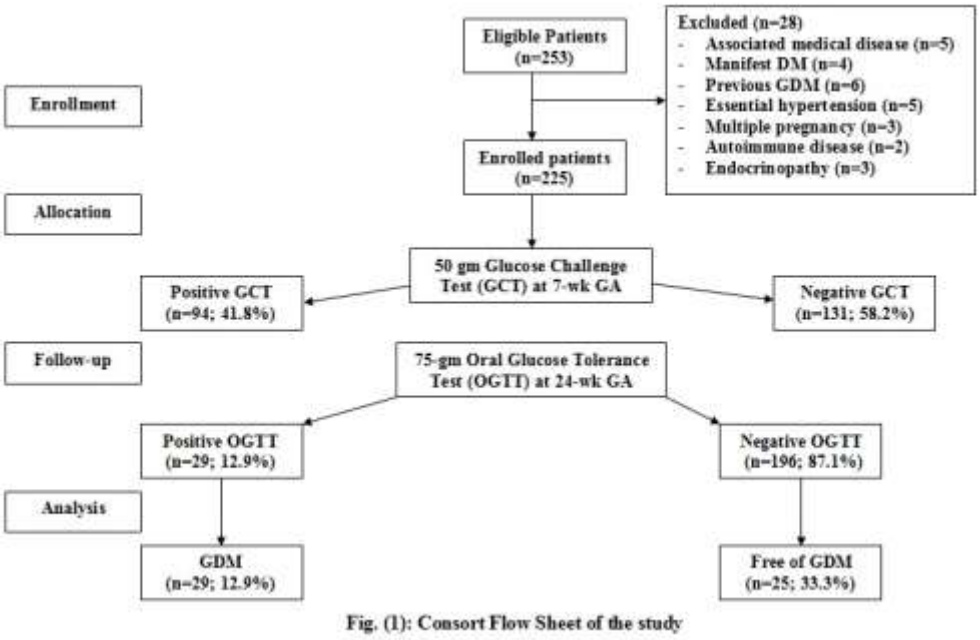

Fig. (1): Consort Flow Sheet of the study

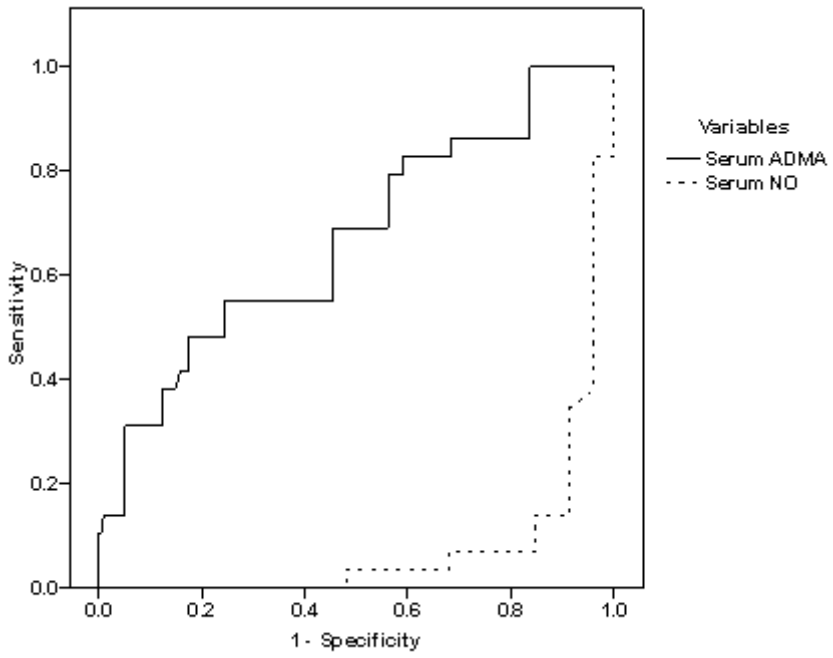

Fig. 2:- ROC curve analysis for baseline serum ADMA and NO levels as predictors for development of GDM

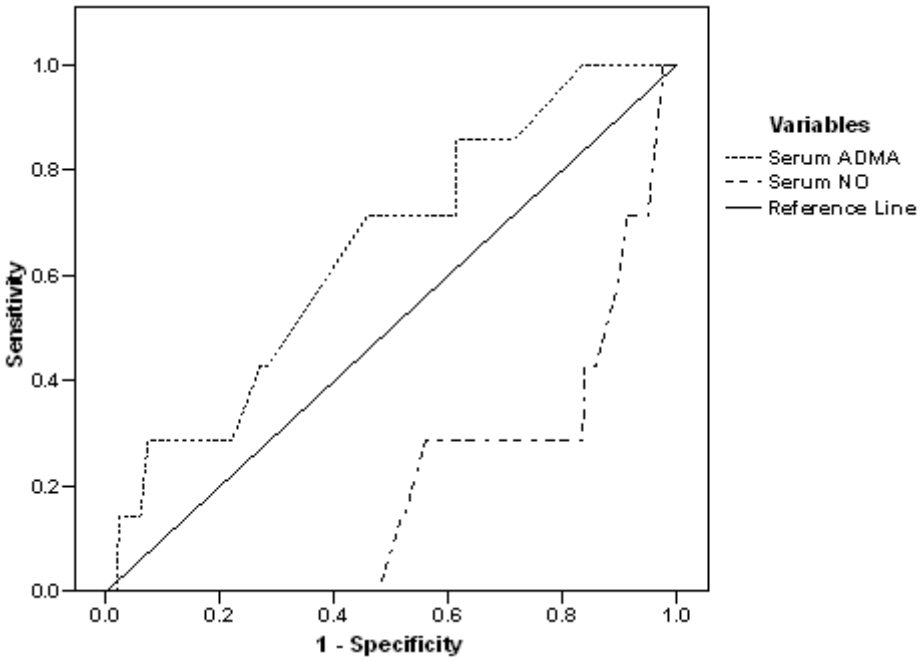

Fig. 3:- ROC curve analysis for baseline serum ADMA and NO levels as predictors for development of IR manifested as high extent of change in serum insulin levels.

Discussion:-

Pregnancy imposes glucogenic stresses on maternal body as evidenced by the reported frequency of women gave positive 50-GCT and of women had IR early in pregnancy, despite of the concomitant blood glucose levels that were in normal acceptable ranges. Moreover, on the 24th wk GA, all enrolled women showed high blood glucose and serum insulin levels with significantly higher HOMA-IR score compared to baseline estimates. These findings go in hand with multiple studies previously documented the glucogenic effect of pregnancy and attributed it to disturbed regulatory mechanisms, where **Giacobbe et al.** [25] found high mobility group box 1, a marker of chronic inflammation, is associated to GDM and IR during pregnancy, while **Silva et al.** [26] documented that a defective signalling via Akt/mTOR in response to insulin is a central and common mechanism of IR in GDM. Also, **Tsiotra et al.** [27] suggested that adipose tissue expression of adipokines contribute to the increased IR and low-grade inflammation that characterizes GDM. Moreover, **Hill** [28] attributed peripheral IR during pregnancy to increasing maternal levels of placental variant growth hormone and the progressively increasing insulin levels to the counter-balance by an increase in insulin availability through an expansion of maternal pancreatic β-cell mass.

Furthermore, at 24th week GA, 29 women (12.9%) had abnormal 75-OGTT and considered as having GDM as they showed significantly higher fasting and postprandial blood glucose, higher serum insulin and HOMA-IR score. In line with the reported figure for GDM, **Sacks et al.** [29] found the overall frequency of GDM was 17.8% and ranged between 9.3 and 25.5% among centers that participated in HAPO Study using the IADPSG criteria and **Waters et al.** [30] out of meta-analysis found 14.3% of studied pregnant women had GDM defined according to IADPSG criteria. Also, **Lacaria et al.** [31] and **Du et al.** [32] reported prevalence rates of GDM of 10.9% and 8.5%, respectively.

The reported frequency of women developed GDM among study population of women who had no previous GDM or manifest DM and despite of the high frequency of women gave positive GCT indicated susceptibility of certain women to develop GDM, irrespective of the history and result of GCT. In support of this suggestion, **Wang et al.** [33] indicated that miR-binding-single nucleotide polymorphisms in T2DM candidate loci were associated with GDM susceptibility and supposed that a similar genetic basis for GDM and T2DM.

Development of GDM was positively and significantly correlated with baseline body weight and BMI, a finding spotting light on the role of pre-conception obesity on increasing the liability for development of GDM. Similarly, **Pan et al.** [34] reported that women with GDM were older and had higher BMI than women free of GDM and

Zambon et al. [35] documented that obesity amplify the inflammatory and oxidative status during pregnancy resulting in increased local and systemic biomarkers particularly when GDM is diagnosed. Also, **Lindsay et al.** [36] reported a high rate of IR among pregnant women of moderately older age and high rate of obesity especially if diagnosed late in gestation. Moreover, trials to minimize such effect of BMI were disappointing as **Du et al.** [32] found diet pattern either Western or traditional during pregnancy may increase the risk of GDM and **Lindsay et al.** [36] tried dietary advice and glucose monitoring to evaluate its impact on IR and reported improvements in blood glucose levels but were insufficient to significantly affect IR.

Baseline serum ADMA and NO levels were significantly correlated with baseline body weight and BMI, presence of GDM, and HOMA-IR score. All of these correlations were positive with ADMA and negative with NO. Similarly **Atay et al.** [37] found NO, glucose, creatinine, and microalbuminuria were significantly different between GDM patients and control subjects and in another study, **Atay et al.** [38] found normotensive patients with GDM had significantly reduced NO levels. Moreover, **Gumus et al.** [39] found the insulin and ADMA, and HOMA-IR levels were significantly higher in patients with a history of GDM and **Xia et al.** [40] detected significantly higher serum ADMA and CRP with increased carotid intima-media thickness (CIMT) in women with previous GDM than healthy controls with positive correlation between ADMA and CRP and CIMT with ADMA being a significant predictor for elevated CIMT in subjects with previous GDM. In trial to explore the pathogenesis of low serum NO in women with GDM, **Anaya et al.** [4] found endothelial cells of intact vessels show sustained Ca^{2+} bursting which drive prolonged NO production in response to ATP, while in both GDM and DM pregnancies, despite of similar incidence of Ca^{2+} bursts there is a reduction in NO output.

ROC curve analysis defined low baseline serum NO and high BMI and serum ADMA as significant predictors for development of GDM with high baseline serum ADMA is specific, while low baseline NO is sensitive predictors. These findings go in hand with **Pleiner et al.** [41] who found obesity, IR and increased ADMA levels appear to be strong contributors to endothelial dysfunction observed in women with GDM. Also, **Sertkaya et al.** [42] detected significantly higher plasma ADMA level in cases with abnormal GCT but normal OGTT and in GDM than in pregnant women with normal GCT and **Sciacqua et al.** [43] reported that ADMA was strongly related to endothelial dysfunction and newly diagnosed T2DM patients, free of clinically manifest vascular complications showed higher ADMA and L-arginine values compared to normal subjects.

Considering high serum insulin in conjunction with high blood glucose is a marker for IR, the extent of serum insulin change showed positive significant correlation with baseline serum ADMA and negative significant correlation with baseline serum NO, but ROC curve analysis showed that low baseline serum NO is a significant predictor IR. Such relationship between NO level and IR could be attributed the fact that insulin metabolic signaling increases endothelial cell NO production, so impaired vascular insulin sensitivity is an early defect leading to impaired vascular relaxation through reduction of NO production by endothelium [44, 45]. Moreover, IR induces pathway-specific impairment in phosphatidylinositol 3-kinase-dependent signaling leading to imbalance between NO production and secretion of endothelin-1 and induces endothelial dysfunction [46] which is characterized by reduced production of NO, thus creating a vicious circle [47].

In line with the reported correlations between IR on one side and both of ADMA and NO on the other side as a marker for endothelial dysfunction, **Sertkaya et al.** [42] found ADMA level was significantly correlated with IR indices and with 2-h insulin level which was the independent influencing constant for ADMA and attributed the elevated ADMA level to elevated insulin level. Thereafter, **Al-Assar et al.** [48] out of their clinico-experimental trial found increased ADMA and up-regulated arginase contribute to endothelial dysfunction as determined by the presence of IR in human obesity, most probably by compromising arginine availability.

Conclusion:-

Pregnancy imposes maternal glucogenic stress associated with IR, irrespective of fasting blood glucose and may progress to manifest GDM with a frequency of 12.9%. IR induces endothelial dysfunction manifested as low serum NO and high serum ADMA that seems to aggravate the detected IR. Baseline serum NO could predict the extent of change of serum insulin at the 24^{th} week GA.

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