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RESEARCH ARTICLE

ASSESSMENT OF ENDOTHELIAL HEMOSTASIS; SERUM NITRIC OXIDE AND ENDOTHELIN-1 LEVELS IN ISCHEMIC CEREBROVASCULAR STROKE WITH OR WITHOUT TYPE 2 DIABETES.

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Key words:-

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Abstract

Background: stroke is the most common cause of disability as well as the second leading cause of mortality worldwide. The role of endothelial dysfunction in stroke is critical. Nitric oxide (NO) and endothelin-1 (ET-1) which are produced in endothelial cells are leading molecules regulating vascular function. **Aim of the work:** the aim of our study was to estimate serum nitric oxide and endothelin-1 levels as biomarkers of endothelial function in ischemic cerebrovascular stroke with or without type 2 diabetes mellitus (T2DM).

Methods: A cross-section study included 100 patients with ischemic stroke who were stratified into two groups according to their fasting blood glucose into non-diabetic group (n=45) and diabetic group (n=55). The levels of serum NO were determined calorimetrically. Serum ET-1 concentrations were also estimated by enzyme immunoassay technique.

Results: Our results showed that non diabetic patients with ischemic stroke had significantly higher values of serum NO compared to diabetic group. On the contrary; there was highly significant elevated serum ET-1 levels in diabetic group compared to non-diabetic group. After adjusted for the traditional risk factors, logistic regression analysis test demonstrated that both serum NO and ET-1 levels were statistically significant predictors of insulin resistance among patients with ischemic stroke. Linear regression analysis test showed that serum NO levels were independently correlated with high density cholesterol (HDL.C) values and systolic blood pressure. Regarding serum ET-1 levels, they were independently correlated with homeostasis model assessment of insulin resistance index (HOMA-IR) and waist/hip ratio (WHR) in patients with ischemic stroke.

Conclusions: Both serum endothelin-1 and nitric oxide levels could be useful diagnostic biomarkers predicting insulin resistance among patients with ischemic stroke.

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1391

Introduction:-

Stroke is defined as rapidly developing clinical symptoms and/or signs of focal, and at times global loss of brain function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin [1]. There is much evidence suggesting that stroke is the most common cause of disability as well as the second leading cause of mortality in the world [2,3].

Remarkably, diabetes is increasing in epidemic proportions globally in the third world countries. In Egypt, the prevalence of diabetes in adults is 14.9%, and there are over 7.8 million cases of diabetes in Egypt in 2015. Moreover, the number of undiagnosed diabetic patients is more than 3.2 million according to International Diabetes Federation (IDF), 2015 [4]. Patients with diabetes always show impaired endothelial function, this is somewhat due to the frequent association of the disease with other cardiovascular risk factors, such as hypertension, obesity, and dyslipidemia as well[5].

Endothelial dysfunction is characterized by a shift in the actions of the endothelium toward reduced vasodilatation, a pro-inflammatory state, and prothrombotic properties as well. There is much evidence suggesting that endothelial dysfunction can play a role in the pathogenesis of ischemic stroke [6]. It is associated with most forms of cardiovascular diseases. Free radicals can damage the endothelium, and leave it overly permeable, allowing toxins to pass into body tissues [7].

NO is a pluripotent regulatory gas in the vascular system. Endothelial derived NOS (eNOS) plays an important role in maintenance of vascular homeostasis, including regulation of cerebral circulation [8].

Endothelin (ET) is a bioactive peptide produced by endothelial cells that can constrict vessels vigorously. It can also enhance the constriction of myocardium and smooth muscles, as well as promote neuroendocrine role[9]. ET is a powerful pro-differentiation agent and a cell-growth factor that can promote cell mitosis, participate in tumor growth and induce mitosis in tumor growth as well [10]. Three types of ET have been identified, ET-1, ET-2 and ET-3, of which ET-1 is the most potent biomolecule. It has been also shown that ET-1 plays a major role in the regulation of the pathogenesis of malignant tumors [11].

The burden of stroke and other cardiovascular diseases is raising in low and middle income countries, where especially, prevention remains the most cost-effective means of intervention. Noteworthy, there are few studies about the correlations between endothelial hemostasis and ischemic cerebrovascular stroke. Thus the aim of our study was to estimate serum nitric oxide and endothelin-1 as biomarkers of endothelial hemostasis in ischemic cerebrovascular stroke with or without type 2 diabetes mellitus (T2DM) and to assess the correlation between them and clinical-laboratory features of stroke.

Subjects and Methods:-

A cross-section study included 100 patients with ischemic stroke recruited from Internal Medicine and Neurology Departments; Zagazig University Hospitals. Patients were stratified into two subgroups according to their fasting blood glucose levels based on the American Diabetes Association(ADA), criteria reported in 2015 (**American Diabetes Association, 2015**). Those without T2DM (n= 45), and 55 patients with T2DM. All subjects were matched, as regard age, gender, and ethnic origin.

The patients were chosen with the following inclusion and exclusion criteria:

[I] Inclusion criteria: Focal or global neurological deficit lasting > 24 hours on initial neurological evaluation, CT scan of the brain showed evidence of cerebral ischemia.

[II] Exclusion criteria: Non-ischemic etiology such as hemorrhagic stroke (patients with intracerebral hemorrhage, subarachnoid hemorrhage), patients who had received drugs known to affect the level of ET-1 or NO, such as glucocorticoids, NSAID, nitrate, beta-blockers or heparin, cases with history of respiratory disease, cancer, severe hepatic, renal diseases, acute illness, hormonal therapy, any active inflammatory diseases, alcoholism, carotid artery surgery and chronic heart failure were excluded from the study.

All patients in the study were subjected to the following: thorough history taking, full clinical assessment including general and neurological examination. Stroke severity within 72 hours of onset of symptoms was assessed using the National Institute of Health Stroke Scale (NIHSS) [12]. CT scan of the brain was performed for each patient to exclude intracranial hemorrhage and to diagnose cerebral infarction including its site and size. If CT scan was negative, it was repeated after 72 h. The size of the lesion was calculated according to the formula $0.5 \times A \times B \times C$ [where A & B are the largest perpendicular diameters measured on CT and C is the slice thickness (10 mm)] [13]. All scans were performed on Siemens Somatom Balance scanner (Siemens Company, Germany).

Full laboratory investigations were carried out including (Complete blood count, erythrocyte sedimentation rate, blood glucose level, lipid profile, liver and kidney function tests. In addition, ECG, transthoracic echocardiography, and carotid duplex were performed as part of stroke workup.

Blood sampling:-

Blood samples were drawn from all subjects after an overnight fast and divided into 3 portions: 1 ml of whole blood was collected into evacuated tubes containing EDTA, for hematocrit, HbA1c. The second ml of whole blood was collected into evacuated tubes containing potassium oxalate and sodium fluoride (2:1) for fasting blood glucose. Sera were separated immediately from remaining part of the sample and stored at -20°C until analysis.

Biochemical assays:-

We measured fasting blood glucose using the glucose oxidase method (Spinreact, Girona, Spain). Total cholesterol, HDL cholesterol, and triglycerides were measured by routine enzymatic methods (Spinreact, Girona, Spain). LDL cholesterol was calculated by Friedewald formula [14].

Fasting serum insulin concentrations were measured using high-sensitivity enzyme-linked immunosorbant assay (ELISA). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated.

Estimation of serum NO and serum ET-1 levels:-

Serum NO levels were measured using colorimetric method of Montgomery and Dymock, by kit purchased from Biodiagnostic (Egypt). Serum ET-1 levels were estimated using a quantitative sandwich ELISA method according to manufacturer's instructions (R& D Minneapolis, MN, USA) ELISA kit.

Ethical consideration:

The ethical committee of Faculty of Medicine, Zagazig University approved our study protocol, and all participants assigned written informed consent.

Statistical analysis:-

Statistical analyses were performed using the Statistical Package for the Social Sciences for Windows (version 19; SPSS Inc., Chicago, IL, USA). Continuous data were expressed using (mean \pm standard deviation) and were analyzed using t test. One-way analysis of variance (ANOVA) test was done to compare different parameters between more than two groups. Receiver operating characteristic (ROC) analysis was performed to assess sensitivities, specificities, area under the curve (AUC), and the cutoff values of NO as well as ET-1 for diagnosis of T2DM among patients with ischemic stroke, linear regression analysis was done to detect the main predictors of NO and ET-1 in patients with ischemic stroke. Logistic regression analysis was performed to determine the predictor markers associated with T2DM among patients with ischemic stroke. We considered P to be significant at <0.05 .

Results:-

Clinical and biochemical characteristics of the studied groups as summarized in Table 1

There were significant higher values of LDL-cholesterol in female group compared to male group, however, there were non-significant differences regarding other parameters ($p > 0.05$).

Table 1:- clinical, anthropometric and laboratory characteristics of studied subjects.

	Male group (mean \pm SD),(n=60)	Female group (mean \pm SD) ,(n=40)	P
Age (years)	59.45 \pm 14.012	57.13 \pm 17.3	0.686
Systolic blood pressure (mm Hg)	131.76 \pm 7.82	134.03 \pm 11.2	0.239
Diastolic blood pressure (mm Hg)	86.36 \pm 4.58	87.70 \pm 5.33	0.185
Body mass index (kg/m ²)	29.2 \pm 11.15	30.1 \pm 10.60	0.697
Waist/hip ratio	0.962 \pm 0.234	0.98 \pm 0.17	0.569
Total cholesterol (mg/dL)	191.75 \pm 36.6	200.67 \pm 43.9	0.274
Triglycerides (mg/dL)	173.6 \pm 41.1	181.4 \pm 43.47	0.365
LDL -C (mg/dL)	109.85 \pm 26.4	120.4 \pm 24.3	$<0.05^*$
HDL -C (mg/dL)	47.96 \pm 8.47	47.19 \pm 10.3	0.681
Fasting blood glucose (mg/dL)	135.5 \pm 62.2	118.1 \pm 46.7	0.135
Fasting serum insulin	25.26 \pm 22.2	29.9 \pm 27.2	0.352
HOMA-IR	9.24 \pm 8.72	9.24 \pm 8.72	0.467
HbA1c (%)	6.378 \pm 1.55	6.1 \pm 1.32	0.373

HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessments of insulin resistance; HbA1c, hemoglobin A1c cholesterol,* $P < 0.05$.

Clinical anthropometric and laboratory parameters in ischemic stroke subgroups:-

As shown in Table 2, diabetic cases group had significantly higher mean values of systolic blood pressure, fasting blood glucose, fasting serum insulin, HbA1c, HOMA-IR and TG than in non-diabetic group. Moreover, type 2 diabetic patients had significant higher values of body mass index and waist/hip ratio ($p < 0.001$) compared to non-diabetic group. On the contrary, there was a significant lower value of HDL-cholesterol in non-diabetic group compared to diabetic group ($p < 0.001$). There were non-significant differences regarding other parameters ($p > 0.05$).

Table 2:- Laboratory and anthropometric parameters in patients with ischemic stroke stratified according to fasting blood glucose.

	Non-diabetic group (mean± SD) (n=45)	Diabetic group (mean± SD) (n=55)	P
Age (years)	59.45±14.01	57.13±17.3	0.342
Body mass index (kg/m ²)	24.8±11.7	33.5±8.42	<0.001*
Waist/hip ratio	0.89±0.29	1.03±0.06	<0.001*
Systolic blood pressure (mm Hg)	128.2± 7.176	136.3±9.4	<0.001*
Diastolic blood pressure (mm Hg)	87.58± 4.92	86.06± 4.8	0.126
Total cholesterol (mg/dL)	206.1± 40.6	182.1± 34.6	<0.001*
Triglycerides (mg/dL)	187.2±41.1	206.1± 40.7	<0.001*
LDL .C (mg/dL)	112.3±29.2	115.5± 23.2	0.534
HDL .C (mg/dL)	53.7±4.33	42.6±9.14	<0.001*
Fasting blood glucose (mg/dL)	84.6±7.6	164.4± 54.5	<0.001*
Fasting serum insulin (mg/dL)	131±5.27	38.6±27.58	<0.001*
HbA1c (%)	5.4±1.47	6.9±1.02	<0.001*
HOMA-IR	2.92±0.984	13.22± 7.02	<0.001*

HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein; HOMA-IR, homeostasis model assessments of insulin resistance; HbA1c, hemoglobin A1c cholesterol,* $P < 0.05$.

Comparison of serum NO ($\mu\text{mol/l}$) and plasma ET-1(pg/ml) levels among the studied groups (Fig.1, 2)

Non diabetic patient with ischemic stroke had significantly higher values of serum NO ($2.47 \pm 0.19 \mu\text{mol/l}$) compared to diabetic group ($1.94 \pm 0.41 \mu\text{mol/l}$) (Fig .1). On the contrary, there was highly significant elevated serum ET-1 level in diabetic group ($9.5 \pm 2.16 \text{pg/ml}$) compared to non-diabetic group ($6.41 \pm 1.98 \text{pg/ml}$) (Fig .2).

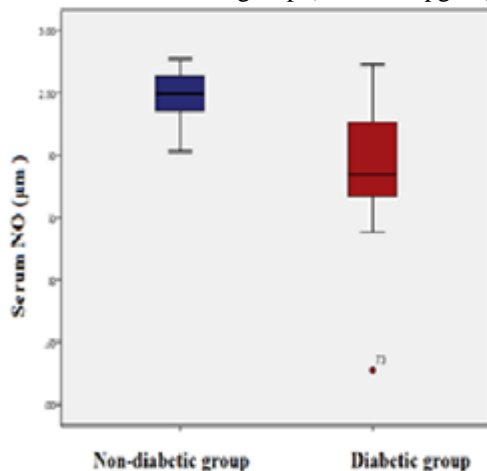


Figure 1

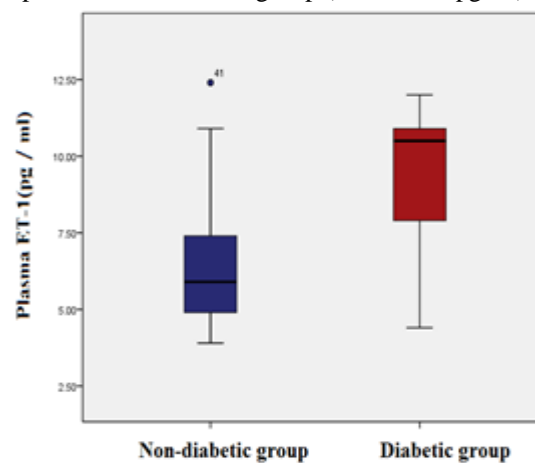


Figure 2

Linear regression analyses in patients with ischemic stroke

Linear regression analysis was done to assess the main independent parameters associated with serum ET-1. Our results showed that, plasma endothelin-1 levels were independently correlated with HOMA-IR and Waist/hip ratio ($p < 0.001$) (Table 3).

Table 3:- Linear regression analysis to test the influence of the main independent variables against endothelin-1 pg/ml (dependent variable). * P < 0.05

Model	Unstandardized Coefficients		Standardized Coefficients	t	p	95% C. I	
	β	SE	Beta			Lower Bound	Upper Bound
(Constant)	2.184	5.107		0.428	0.670	7.939	12.240
LDL.C	0.002	0.008	0.021	0.259	0.796	0.018	0.013
HDL.C	-0.030	0.037	0.107	0.810	0.420	0.101	0.044
Fasting blood glucose	0.001	0.004	0.029	0.357	0.722	0.005	0.009
HOMA-IR	0.205	0.048	0.580	4.246	<0.001*	0.109	0.293
Waist/hip ratio	7.103	2.893	0.203	2.455	<0.001*	1.359	12.383
Body mass index	0.008	0.028	0.030	0.302	0.764	0.064	0.045
Systolic blood pressure	0.009	0.028	0.034	0.340	0.735	7.939	12.240

Linear regression analyses in patients with ischemic stroke:-

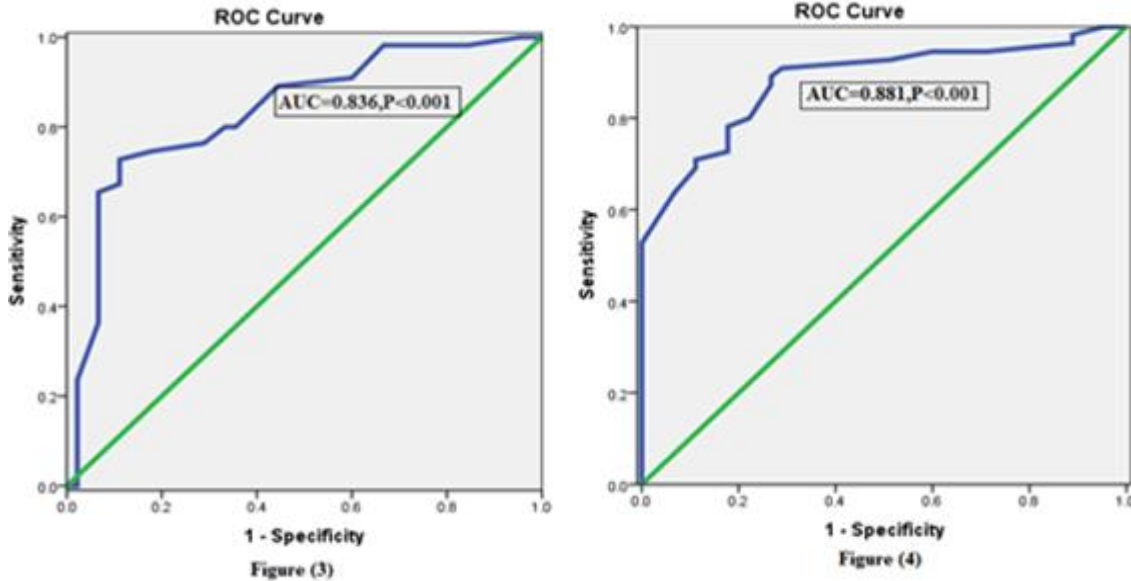
Linear regression analysis was done to assess the main independent parameters associated with serum nitric oxide. Our results showed that serum nitric oxide levels were independently correlated with HDL-cholesterol and systolic blood pressure ($p < 0.001$) (**Table 4**).

Table 4:- Linear regression analysis to test the influence of the main independent variables against serum nitric oxide (μm) (dependent variable).

Model	Unstandardized Coefficients		Standardized Coefficients	t	p	95% C. I	
	β	SE	Beta			Lower Bound	Upper Bound
(Constant)	0.118	0.077		1.522	0.131	0.272	0.036
LDL.C	0.000	0.000	0.043	1.919	0.058	0.000	0.000
HDL.C	0.016	0.001	1.007	27.74	<0.001*	0.015	0.017
Fasting blood glucose	7.408	0.000	0.029	1.339	0.184	0.000	0.000
HOMA-IR	0.000	0.001	0.013	0.355	0.723	0.002	0.001
Waist/hip ratio	0.058	0.044	0.030	1.326	0.188	0.145	0.029
Body mass index	0.000	0.000	0.024	0.887	0.378	0.001	0.000
Systolic blood pressure	0.001	0.000	0.067	2.472	<0.001*	0.000	0.002

Accuracy of plasma endothelin-1 and serum nitric oxide for discriminating diabetic from non-diabetic patients with ischemic stroke by ROC Analyses:-

The cut-off values of plasma ET-1 and serum nitric oxide levels were determined by ROC to discriminate diabetic from non-diabetic patients with ischemic stroke; they were 8.65 and 2.3, and the AUC were 0.836(95% CI 7.45–9.17, $P < 0.001$) and 0.881 (95% CI 0.814–0.948, $p < 0.001$, respectively). The sensitivities and the specificities of endothelin-1 were 72.9 % and 99.8 % and nitric oxide serum levels were 80.1% and 77.8%, respectively (Fig. 3, 4).



Logistic regression analysis evaluating the association of serum NO and ET-1 with T2DM among patients with ischemic stroke (Table 5)

After adjusted for the traditional risk factors, logistic regression analysis test was done to evaluate the predictors of insulin resistance among patients with ischemic stroke, serum NO and serum ET-1 were statistically significant predictors of insulin resistance among patients with ischemic stroke ($p < 0.001$).

Table 5:- Logistic regression analysis of the main clinical and biochemical predictors of diabetic patients with ischemic stroke.

	B	S.E.	Wald	P	odds	95% C.I	
						Lower	Upper
Constant	5.246	5.757	0.830	0.362	189.890		
Serum ET-1(pg/ml)	.275	.136	4.114	0.043	1.317	1.009	1.718
Serum NO ($\mu\text{mol/l}$)	13.075	3.710	12.419	0.000	0.000	0.000	0.003
Body mass index (kg/m^2)	0.030-	0.043	0.484	0.487	0.970	0.891	1.056
LDL-Cholesterol (mg/dl)	0.006-	0.012	0.274	0.601	0.994	0.971	1.017

ET-1, endothelin-1 ; NO, nitric oxide levels ; LDL-C, low-density lipoprotein* $P < 0.05$.

Discussion:-

There was great evidence that the etiology of ischemic stroke is known to be a multifactorial disorder in addition to the commonly accepted risk factors [15]. Additionally, increasing evidences suggested endothelial dysfunction role in the pathophysiology of ischemic stroke. Endothelial dysfunction can be found to be both macro- and microvascular, which leads to an imbalance between vasodilation and vasoconstriction capability in homeostasis regulation [16-19]. ET-1 being a potent vasoconstrictor, increases, while NO, as vasodilator substance, decreases in endothelial dysfunction. The vasoconstrictive effect of ET-1 is more prominent on microvascular wall including subendocardial compared to macrovascular. Moreover, an increase in ET-1 causes an endothelial dysfunction [20-21].

Patients with diabetes mellitus are at markedly increased risk of death due to cerebrovascular disease, and this is true of both T1DM and T2DM [22]. In fact, T2DM patients make up the vast majority of diabetic stroke (97% in the Nurses' Health Study) [23].

Early diagnosis and management of endothelial dysfunction in ischemic stroke especially in diabetics is very important. To address this need, we have focused on biomarker of endothelial dysfunction, especially NO and ET-1. Thus the aim of our study was to investigate the role NO and ET-1 as biomarkers of endothelial hemostasis in ischemic cerebrovascular stroke with or without T2DM and to assess their correlation with clinical-laboratory features of stroke.

Our results showed that, among patients with ischemic stroke, 60 % were males and 40% were females. In comparison between male and female group, our findings confirmed that female group had significantly higher values of LDL-cholesterol. Otherwise all other parameters showed non-significant difference between both groups.

In accordance to our finding, a prospective population-based study, showed an increased relative risk for developing stroke of 1.5 to 2 fold in men and 2 to 6.5 fold in women. This increased risk is seen even early after diagnosis of stroke in newly treated T2DM patients [23].

In our research, we found significant higher levels of systolic blood pressure, fasting serum insulin, fasting blood glucose, HOMA-IR, HbA1c, total cholesterol, triglycerides, body mass index, and waist/hip ratio in T2DM group compared to non-diabetic group.

Similar finding was observed also by **Jeerakathil and his colleagues**; who found higher relative risk of stroke in the T2DM group compared to general population [25]. This can be explained as both diabetic as well as obese patients usually consume a high-calorie diet rich in macronutrients which induce vascular abnormalities [26]. Indeed, protein, lipid, and glucose loads are associated with a marked production of reactive oxygen species (ROS) and high-fat meals, with impaired endothelium-dependent vasodilation [27]. Similar to our finding, several studies detected the impairment of endothelial function in relation to blood glucose levels fluctuation, HbA1c, and insulin resistance [28,29]. In contrast to our results, the study of **Zampetaki et al.**, revealed significantly higher value of low-density cholesterol levels in association with endothelial function in [30].

Increasing evidence suggests that diabetes mellitus, hypercholesterolemia, hypertension, and smoking lead to atherosclerosis as well as endothelial dysfunction [31, 32]. The main finding in the current study that non-diabetic patient with ischemic stroke had significantly higher values of serum NO compared to diabetic group. On the contrary; there were highly significant elevated serum ET-1 levels in diabetic group as compared to non-diabetic group. In agreement with our results, **Manrique et al.** observed that patients with diabetes had endothelial dysfunction. There is a general consensus that hyperglycemia and diabetes lead to impair NO production and damaged vasodilatory activity [33]. **Piconi et al.**, found that chronic hyperglycemia leads to weak integrity and apoptosis of endothelial cell [34]. In light of this fact, several clinical studies have demonstrated an impaired endothelium-dependent vasodilation in conduit or resistance vessels of T2DM patients [35]. ROS produced in diabetic patients also contributes to endothelial injury and impair endothelial repair. They are directly cytotoxic for endothelial cells, react with NO, decrease NO bioavailability, and form peroxynitrite anions which act as powerful oxidants. Lifestyle modification has a potential to increase the number of endothelial progenitor cells and improve their migratory capacity, helping to repair the damaged endothelium [36-38].

The results presented herein are innovative; as this study performs a robust evaluation of NO and ET-1 as diagnostic biomarker of endothelial dysfunction. Both serum ET-1 and nitric oxide could be useful diagnostic biomarkers detect T2DM among patients with ischemic stroke. Interestingly; the power of NO was sensitive and specific parallel to ET-1. Our study explored that after adjusted for the traditional risk factors, serum NO and ET-1 levels were statistically significant predictor of ischemic stroke among patients with ischemic stroke. Endothelial dysfunction seems to precede the development of diabetes, as impaired endothelium-dependent vasodilation was observed in healthy non-diabetic subjects who have a first degree relative with T2DM [32], as well as in subjects with impaired glucose tolerance [31].

Our results showed that, serum NO levels were independently correlated with HDL-cholesterol and systolic blood pressure. In agreement with our results, **Boden and Shulman** found the progression of insulin resistance to T2DM parallels to the progression of endothelial dysfunction to atherosclerosis. Moreover, insulin resistance is closely linked with visceral adiposity, and early data suggested that free fatty acids were responsible for this association [37]. The eNOS acts as a protective role in ischemic stroke by inhibiting platelet aggregation and leukocyte adhesion to vascular endothelium, and thus protects against vascular pathological changes such as vascular muscle cell growth and proliferation [38]. Most importantly, eNOS also protects from atherosclerosis, which is an independent risk factor for ischemic stroke incidence [39-41].

Our results showed that ET-1 concentrations were independently correlated with HOMA-IR and Waist/hip ratio., findings which were similar to results of **Romero et al.** who observed that ET-1 may contribute to the development of endothelial dysfunction, and consequently insulin resistance by increasing the production of ROS, mainly superoxide anion, in the vasculature. This is mainly dependent upon the activation of NADPH oxidase

protein expression and activity [42]. Similar to our finding, **Lee and Poh** reported in their study that ET-1 values were positively correlated with insulin resistance as well as endothelial dysfunction [43].

Conclusion:-

The results of this study reached to a conclusion that serum ET-1 levels were significantly elevated in T2DM patients with ischemic stroke. While serum NO levels were decreased, hence Serum NO and ET-1 could be early predictors of insulin resistance in patients with ischemic stroke.

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