ASSESSMENT OF THE SERUM INTERLEUKIN-6, INTERCELLULAR ADHESION MOLECULE-1, NITRIC OXIDE AND C-REACTIVE PROTEIN IN ACUTE ISCHEMIC STROKE

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ABSTRAT

Background: Cerebral ischemia initiates an inflammatory response in the brain which is a composite process that involves many inflammatory mediators.The investigation of inflammatory response in the acute stage may contribute to improve the treatment of ischemic stroke. **Objective:** We assessed the relationship between peak values of serum interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1),erythrocyte sedimentation rate (ESR), c-reactive protein (CRP)and nitric oxide (NO)in the first week after ischemic stroke, with measures of stroke severity and outcome.**Methods:**forty-five patients (22 males and 23 females) with ischemic stroke were prospectively recruited. Serum IL-6, and other markers of peripheral inflammation, was measured at time points 24 h, as well 7 days after symptom onset.Twenty-five age-matched volunteers were used as controls. We correlated levels with stroke severity assessed by the NIH Stroke Scale; stroke outcome at 1 month assessed by BI and computed tomography (CT) brain infarct volume. **Result(s):** Peak serum IL-6 and CRP concentrations correlated significantly ($p < 0.001$) with CT brain infarct volume ($r = 0.75$) and NIHSS at 1 week ($r = 0.72$). They correlated similarly with clinical outcome at 1 month. Strong associations were also noted between either peak serum ICAM-1 or NO concentrations and short-term stroke outcome. **Conclusion(s):**These data provide evidence that the peripheral inflammatory response may be related to the severity of acute ischemic stroke, and may be used as predictors for clinical outcome.

INTRODUCTION

Accumulating evidence suggests that inflammation plays an important role in the development of cardiovascular and cerebrovascular disease, indicating important interactions between the nervous and immune systems[(1)]. The past few years had been stressed the role of inflammation in the pathophysiology of acute brain ischemia and how most inflammatory events are mediated by cytokines, small glycoproteins expressed by many cell types in response to acute cerebral ischemia[(2)].

Circulating levels of cytokines are found to be related to the prognosis after stroke. Interleukin-6 (IL-6) is one of the most studied cytokines related to inflammation in stroke[(3)].

Other markers of the acute phase response have also been implicated in the pathophysiology and outcome following ischemic stroke. The most studied biomarkers of inflammation C-reactive protein (CRP) is an acute phase reactant protein which is produced predominantly by hepatocytes under the influence of cytokines i.e. IL-6 and tumor necrosis factor-alpha (TNF-α).Raised serum CRP, is thought to be the major risk factor for acute ischemic stroke due to participation in formation of Atherosclerosis in the cerebral vessels in genetically prone patients. It is markedly up regulated in atheromatous plaques where it promote Low density lipoprotein (LDL) cholesterol uptake by macrophages, a key step in atherogenesis[(4)].

Elevated circulating levels of hs-CRP and IL-6 are found after stroke and are correlated with infarct size [(5)]and with poor outcome[(6, 7)].

Cytokine release result in upregulation of adhesion molecules, recruitment and activation of leukocytes, promotion of leukocyte-endothelium interaction, and convertion of the local endothelium to a prothrombotic state [(8)].

Intercellular adhesion molecule-1belongs to the immunoglobulin superfamily of CAMs. It is expressed on leukocytes, endothelial cells, epithelial cells, and fibroblasts [(9)].ICAM-1 has been demonstrated to play an important role in the pathogenesis of ischemic infarction. Recently, upregulation of endothelial ICAM-1 in infracted areas has been found in patients with acute ischemic stroke [(10)].

Whereas in low concentrations (nanomolar range), nitric oxide (NO) plays a physiological role in neuronal signaling, in high concentrations (micromolar range), this molecule is enormously cytotoxic [(11)].The production of NO, itself a free radical, promotes tissue injury, e.g., by reaction with with superoxide anion to produce the extremely toxic peroxynitrite (ONOO-) or by interaction with proteins, transition metals, and iron-sulfur-containing or heme-containing compounds [(12)].Excessive NO generation has been implicated in ischemic neurodegeneration and is currently intensely studied as a therapeutic target in ischemic stroke [(12)].

PATIENTS AND METHODS

Between November 2010 and July 2012,we prospectively analyzed 45 consecutive patients (22 males and 23 females) with first-ever ischemic stroke admitted within 12 hs of the onset of stroke symptoms to the intensive care and stroke units, Neurology Department, Zagazig

University Hospitals. Our exclusion criteria were: TIAs, hemorrhagic stroke, present or recent (2 weeks) infection, organ failure,malignancy, surgery within 3 months, collagen and inflammatory disease; and those on steroids or immunomodulatory drugs were also excluded.Also, we excluded each case for which the time of onset of stroke symptoms was not clear. Written informed consents were obtained .

We also enrolled a group of 25 control volunteers (12 males and 13 females). They were matched with the patients as regards, age, sex and risk factors.Stroke was defined according to the WorldHealth Organization as "rapidly developing clinical signs of focal/global disturbance of cerebral function,with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin".

Cerebral infarction was diagnosed on the basis of history, neurological examination, andneuroimaging (CT or brain MRI). CT scan was performed within 12 hs of admission to exclude patients with stroke mimic or primary intracerebral hemorrhage.All subtypes of ischemic stroke were included.

Clinical history, examination, medications and Oxfordshire Community Stroke Project (OCSP) classification [(14)]were recorded at baseline.Diabetes mellitus (DM) was considered to be a risk factor if there was a self-reported history of diabetes mellitus, treatment with glucose lowering medications or diet prior to stroke onset or elevated fasting blood sugar (FBS) ≥ 6.7 mmol/L (140 mg/dl) [(15)].Criteria for hypertension were: systolic blood pressure over 140 mmHg and diastolic blood pressure over 90 mm Hg on at least two occasions (at least one week a part). Patients who reported history of hypertension or current use of antihypertensive medication were also classified as hypertensive[(16)].Atrial fibrillation was diagnosed by electrocardiography.Smoking status was assessed by interview. Dyslipidemia was considered to be a risk factor if there was abnormal fasting lipid profile as defined by **National Cholesterol Education Program (NCEP)**,[(17)]criteria.

Blood sampling and determination of peripheral inflammatory markers

Venous blood was drawn by venepuncture at admission and 5 to 7 days.Blood was collected in tubes containing EDTA (Sarstedt, UK) for analysis of ESR using an automated Westergren method (Starrsed 3, Mechatronics, Holland; supplied by Vitech Scientific, UK).

The remaining blood for serum IL-6, CRP, ICAM-1 and NO was allowed toclot at room temperature for 1 hour; after centrifugation, the serum was stored at -20°C until used.

Serum IL-6 and sICAM-1 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits following manufacturer's instructions (Boster Immunoleader, Fermont, CA 94538, USA). Serum hs- CRP levels were assessed with '*Turbi-latex*' *kit* using turbidimetric analysis. NO released by cells was measured in serum as nitrite by the Griess reaction.

Definition of peak values

For individual patients, the peak value for each peripheral inflammatory marker was defined as the maximum measured value at presentation and 5 to 7 days [(5)]. Patients who died prior to 5 to 7 days, or for whom data were missing, were therefore not assigned a peak value and were excluded from further analysis.

Assessment of stroke severity and outcome

Stroke severity was assessed using the National Institutes of Health Stroke Scale (NIHSS) score [(18)]at presentation and 5 to 7 days.Functional status during the four weeks following stroke was recorded using the Barthel index (BI) [(19)]. Subsequent functional outcome was assessed using BI at 1 months.

Measurement of CT brain infarct volume:

All patients presented with cerebral stroke were subjected to CT brain within the first 24 hours after stroke onset to exclude patients with stroke mimic or primary intracerebral hemorrhage (PICH).

A second CT brain scan for analysis of the infarction volume was performed at 5 to 7 days, in order to minimize the "fogging" effect seen in the second to third weeks after ischemic stroke.

Statistical analysis

The data were tabulated and statistically analyzed using Statistical Package for Social Science (SPSS)Program version 14.0.0 software package [(20)].The qualitative data were presented in the form of numbers and percentage. The Chi-square X^2 test and T test were used as tests of significance. Significance was considered when p value was less than 0.05.

RESULTS

Demographic characteristics of study participants are presented in Table 1. No Statistically significant differences between cases and controls were found for age and sex (table 1).

Also,no significant difference between them as regard risk factor distribution (table 2).

Stroke characteristics and outcome measures are presented in Table 3.The number of patients at each visit is outlined in Figure 1. Three patients died prior to 1 month assessment leaving 42 patients with no NIHSS or BI available.Stroke topography according to vascular territory involved in the studied stroke patients are presented in table (4). In our study, 38 patients (84.44%) had anterior circulation stroke and 7 patients (15.56%) had posterior circulation stroke.

Concentrations of inflammatory markers between cases and controls in the 1st 24 hr of admission and 5^{th}-7^{th} day after that are presented in Table 5 and 6.It was revealed that the mean values of the inflammatory markers at both time points were higher among the patients compared to the controls and this difference was significant (P=0.011).

The correlation between peak peripheral inflammatory markers and outcome measures are shown in table 7. Peak serum hs-CRP and IL-6 concentrations correlated significantly with CT brain infarct size($p < 0.001$) and BI at 1 month($p < 0.05$). Peak serum hs-CRP and IL-6 concentrations also correlated with stroke severity at admission and at 5 to 7 days ($p < 0.001$).Also, peak ESR, ICAM-1 and NO correlated significantly with BI at 1 month.

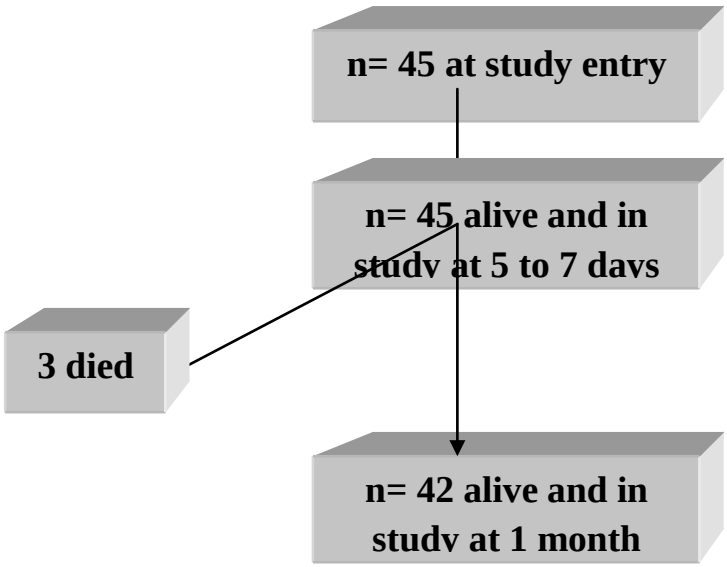

Fig (1).Study profile

Table (1). Age and sex distribution in the two studied groups.

Data \ Groups	Patients (n = 45)		Controls (n = 25)		Test	P
	No.	%	No.	%		
Sex					χ^2	
Male	21	46.7	12	48.0	0.01	0.91
Female	24	53.3	13	52		
Age (years)					t	
Range	18 - 85		20 – 75		0.89	0.37
$\overline{X}$ ±SD	57.2±13.7		54.2±13.2			

Table (2). Distribution of risk factors in the two studied groups.

Risk factors	Patients n =45		Controls n =25		Total n =70		X^2	P
	No.	(%)	No.	(%)	No.	(%)		
Hypertension	31	68.9	15	60.0	46	65.7	0.56	0.45
Diabetes mellitus	23	51.1	7	28.0	30	42.9	3.51	0.06
Dyslipidemia	17	37.8	6	24.0	23	32.9	1.38	0.23
Heart diseases	14	31.1	6	24.0	20	28.6	0.4	0.52
Smoking	6	13.3	3	12.0	9	12.9	0.06	0.83
Contraceptive pills*	10	41.7	6	46.2	16	43.2	0.07	0.79

* Percent of females

NB: more than one risk factor may be present in the same patient

Table (3). Stroke characteristics and outcome measures (n = 45 patients with acute ischemic stroke on admission).

OCSP subtype	**No.**	**%**
Total Anterior Circulation Syndrome	28	62.2%
Partial Anterior Circulation Syndrome	5	11.1%
Lacunar Syndrome	9	20.0%
Posterior Circulation Syndrome	3	6.7%

NIHSS score*	$\overline{X}$ **±SD**	**Range**
Presentation (n= 45)	10.7±4.7	2-23
5 to 7 day survivors (n = 45)	9.2±4.7	0-19
1 month survivors (n =42)	6.5±5.0	0-19

Infarct volume* (cm3) n = 45	5.86±5.6	0.1-22.5

BI*		
1 month survivors (n = 42)	14.9±3.9	6-20

(OCSP) Oxfordshire Community Stroke Project classification
(NIHSS)National Institutes of Health Stroke Scale
(BI) Barthel index

Table (4). Stroke topography according to vascular territory involved in the studied stroke patients.

Stroke topography	**Stroke patients (total no=45)**	
	No.	**(%)**
Anterior circulation stroke	**38**	**(84.44)**
Posterior circulation stroke	**7**	**(15.56)**

Table (5). Comparison between patients and controls regarding the level of the peripheral inflammatory markers in the 1st 24 hour of admission.

Inflammatory markers	**Patients (n = 45)**	**Controls (n = 25)**	**T**	**P**
ESR (mm/hr)	27.1±17.7	12.8±5.6	3.92	<0.001
CRP (mg/L)	18.4±23.6	6.18±1.25	2.59	0.011
IL-6(pg/mL)	20.8±10.1	15.3±4.4	2.54	0.013
ICAM-1(ng/ml)	456.0±182	229.7±70.8	5.94	<0.001
NO (µmol/L)	33.7±16.7	18.9±4.6	4.3	<0.001

Table (6). Comparison between patients and controls regarding the level of the peripheral inflammatory markers in 5^{th}-7^{th} day.

Inflammatory markers	Patients (n = 45)	Controls (n = 25)	T	P
ESR (mm/hr)	33.2±3.4	12.8±5.6	4.86	<0.001
CRP (mg/L)	36.5±32.2	6.18±1.25	4.69	<0.001
IL-6(pg/mL)	26±12.3	15.3±4.4	4.34	<0.001
ICAM-1(ng/mL)	442.3±226	229.7±70.8	4.56	<0.001
NO (µmol/L)	35.8±16.7	18.9±4.6	5.8	<0.001

Table (7). Correlation coefficients between peak peripheral inflammatory markers and outcome measures.

Inlammatory markers	NIHSS at admission	NIHSS at 5-7 days	BI at 1 month	CT brain infarct volume (cm3)
PeakESR (mm/hr)	r=0.25 (p>0.05)	r=0.41 **(p<0.001)**	r=-0.47 **(p<0.001)**	r=0.26 (p>0.05)
Peak CRP (mg/l)	r=0.46 **(p<0.01)**	r=0.42 **(p<0.001)**	r=-0.31 **(p<0.05)**	r=0.46 **(p<0.001)**
Peak IL-6 (pg/ml)	r=0.44 **(p<0.01)**	r=0.37 **(p<0.001)**	r=-0.16 **(p<0.05)**	r=0.65 **(p<0.001)**
Peak ICAM-1 (pg/ml)	r=0.22 (p>0.05)	r=0.33 **(p<0.05)**	r=-0.32 **(p<0.05)**	r=0.66 **(p<0.001)**
Peak NO (µmol/l)	r=0.43 **(p<0.005)**	r=0.52 **(p<0.005)**	r=-0.49 **(p<0.001)**	r=0.14 (p>0.05)

DISCUSSION

Accumulating evidence suggests that inflammation plays an important role in the development of cardiovascular and cerebrovascular disease[21].The central role of inflammation, expressed by cytokine cascade in the pathogenesis of neuronal ischemic damage, could probably represent a chance to recognize possible serum markers of ischemic stroke useful to a diagnostic algorithm of acute ischemic stroke. For this reason, the aim of the present study was toassess the peak serum immunoinflammatory markers in the 1^{st} week of acute ischemic stroke. Also, to detect their relationship with ischemic stroke size and severity as a predictor of short-term outcome in the 1^{st} month of acute ischemic stroke.

We reasoned that a good estimate of the magnitude of the peripheral inflammatory response would be obtained by taking the peak value of measurements within the first 24 hours of stroke, or at the end of the first week. We accept that it is difficult to determine the true peak without serial measurements. Furthermore, only patients with data at all time points, and those surviving to day 5 to 7 could be included in analysis of peak values, therefore excluding patients that died within the first week who may have had the most severe strokes. Previous studies have reported peak values of plasma IL-6 within the 1^{st} week of stroke and confirm the strong correlation between peak plasma IL-6 [5, 22, 23], peak plasmaerythrocyte sedimentation rate *(*ESR) and high-sensitivity C-reactive protein (hs-CRP) [5]with CT infarct volume, stroke severity or clinical outcome. Furthermore, to our knowledge, we may be the first to report association between individual peak values of serum sICAM-1 and NO concentrations within the first week of ischemic stroke, and CT brain infarct size, severity or outcome.

Inflammation is considered as one of the high risk factors for stroke in the initiation, progression and maturation of atherosclerosis. This relationship between ischemic stroke and inflammatory responses has been estimated through the elevation of serum levels of acute phase proteins including IL-6, ESR and hs-CRP [24]. Controversy exists as to whether the release of pro-inflammatory cytokines after focal cerebral ischemia indicates a pathogenic step leading to tissue necrosis or simply reflects the amount of ischemic brain injury. IL-6 concentrations similar to ESR and hs-CRP might merely reflect an acute phase reactant [5,6,25].

An important feature of the present study design is the individual matching of patient and control subjects for age, sex and degree of

atherosclerosis, all of which are related to differences in peripheral inflammatory markers [26, 27].Since atherosclerosis is a known inflammatory stimulus [28], our control population is likely to have elevated inflammatory markers compared with an entirely normal population lacking atherosclerosis, which may be expected to reduce differences between control subjects and patients.

The main finding of this study is that pro-inflammatory cytokine level reflects the inflammatory response in acute brain ischemia and may be associated with stroke severity and outcome. More specifically, the pro-inflammatory IL-6 measured 12 h and 5-7 days after the onset of cerebral ischemia was increased in stroke patients relative to controls and its peak level was associated with the extent of brain damage, more sever stroke and worse stroke outcome. Our data on the elevated level of IL-6 are consistent with other results [24, 29-31].Moreover, correlation of serum IL-6 levels with stroke severity, stroke outcome and extent of brain damage was also found in recent studies that have also shown that high serum levels of IL-6 was correlated with severe stroke, early neurological deterioration and worse outcome [32-34], implicating that this peripheral inflammatory marker may provide useful prognostic information for the clinical course and functional outcome of acute ischemic stroke [5].It is speculated that upregulated IL-6 may be considered to act on the vascular endothelium to increase harmful mediators and mediate inflammatory cascades leading to the aggravation of cerebral ischemic damage [35, 36].The precise mechanism of the association between elevated IL-6 levels and poor clinical outcome is unclear. IL-6 up-regulates the expression of adhesion molecules, such as sICAM-1, selectins and integrins on endothelial cells, leukocytes and platelets, during cerebral ischemia, which leads to secondary neuronal damage [37].

In keeping with IL-6 findings,serum hs-CRP was significantly raised in acute stroke patients and showed to be correlated with stroke size, severity and predictive of short-term stroke outcome. ESR showed significant elevation in stroke patients, without any correlation with stroke severity.**Smith et al.** [5]have studied the predictive value of Peak CRP and ESR measurement in Thirty-sevenconsecutive ischemic stroke patients. Based on the results of their study they have concluded that peak CRP level is strongly associated with the outcome measures. Also, **Abubakar et al.** [38]have also observed significantly positive correlation between hs-CRP and stroke severity at presentation and 30-day post-stroke outcome. The association between increased hs-CRP level and poor outcome after ischemic stroke has been explained by **Muir et al.** [39]who stated that CRP concentration may indicate underlying unstable atherosclerotic disease, CRP concentration may reflect the degree of stroke severity correlating with the degree of inflammation directly consequent to cerebral infarction and CRP may be raised as a consequence of secondary complications of stroke at time of sampling. In clinical practice we may consider high CRP as a "red flag" marker of high mortality, but therapeutic implications of this finding remain uncertain [15].

In different autopsy studies based on the immunocytochemical technique, the expression of sICAM-1 on the surface of damaged cells was assessed during the first week after acute brain ischemia. sICAM-1 was observed on the epithelial cells [40, 41].The assessment of adhesion molecule levels in patients with cerebrovascular diseases needs special caution because of the association with risk factors for atherosclerosis development [42, 43].The presence of atherosclerotic changes is associated with chronic endothelium activation which can provoke the over-expression of cell adhesion molecules [44, 45].

The results obtained in our study confirm that the immune system plays an important role in the ischemic cascade that is initiated after an acute ischemic event. sICAM-1 measured 12 h and 5-7 days after the onset of cerebral ischemia was increased in stroke patients relative to controls. **Matusik et al.**[46]found a significant increase of the sICAM-1 level on the 1st, 5th and 14th day of stroke compared with an age and atherosclerosis risk factors matched control group. **Rallidis et al.** [1]demonstrated significantly higher sICAM-1 levels in ischemic stroke than in healthy controls.However, **Fassbender et al.**[47] **and Supanc et al.** [48]found no significant difference in the concentration of sICAM-1 in patients with acute ischemic stroke and control groups. Possible explanations for contradictory results include difference in the selection criteria for the patients (use of medications influencing expression of adhesion molecules such as aspirin and NSAID's), the differences between control groups, laboratory tests and time frames of blood sampling.

The results of our study suggested that sICAM-1 can be a good molecular marker of neurological deficit in stroke patients. We observed the association of higher peak serum sICAM-1 concentration with greater degree of patient disability and poor short term functional

outcome in 1 month assessed with BI (P< 0.05). The prognostic value of sICAM-1 on in-hospital mortality in patients with ischemic stroke was examined recently by **Rallidis et al.**[(1)]. They found that high sICAM-1 level, determined within the first 12 h of stroke, was associated with early death in middle-aged patients.

The present findings provide evidence that biochemical changes related to NO metabolism and oxidative stress may be considered as markers of tissue injury in brain ischemia.Serum NO was significantly raised in acute stroke patients and predictive of short-term stroke.

Such as, an observed increase in serum NO levels in our patients matches the observation of**Ozkul et al.** [(49)]who found higher serum levels of NO detected in the 70 acute stroke patients in the first 48 h post-stroke as compared to age and risk-matched controls without a history of stroke. They also suggested that NO level was higher in more severe acute cerebral infarcts as patients with less neurologic deficit had lower NO levels.Similarly, **Aygül et al.** [(50)]demonstrates that NO levelswithin 4 days after ischemic strokein plasma and CSF are higher in patients with ischemic stroke than in healthy controls but they disagree with our results as they found no correlation between infarction volume or neurological deterioration and outcome with NO levels. He argued that the lack of correlation between NO and infarction volume or early neurological deterioration may be due to the small sample size.

CONCLUSION

We concluded that the employment of ESR, hs-CRP and IL-6 as early stage biomarkers of acute ischemic stroke could indicate the presence of an acute phase response. Also, we have demonstrated significant associations between acute elevation of sICAM-1 (blood markers of endothelial cell activation) and ischemic stroke. Peak level of circulating sICAM-1 are associated with a poor short-term prognosis in acute ischemic stroke patients.Our data supported the hypothesis of the deleterious effects of oxidative stress on clinical severity and outcome in acute ischemic stroke.

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