ORIGINAL ARTICLE

TNF-ALPHA: A RISK FACTOR FOR ISCHEMIC STROKE

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Background: Inflammatory markers are being explored to aid in stroke diagnosis especially to differentiate between clinical varieties of stroke. This study aimed to compare plasma tumour necrosis factor-alpha (TNF-alpha) and Interleukin-10 (IL-10) levels between stroke patients and controls, as well as between hemorrhagic and ischemic varieties of stroke. **Methods:** Stroke patients who were admitted to Shaikh Zayed Hospital Lahore and Services Hospital Lahore, Pakistan within 24 hours after the onset of stroke symptoms were consecutively asked to participate in this study from June 2011 to December 2011.Venous blood samples were collected within 24 hours of stroke symptoms onset. Plasma TNF-α levels and IL-10 were calculated using commercial enzyme-linked immune-sorbent assay (ELISA). Cytokines levels were dichotomized as detectable yes/no and were compared between different groups using chi square test. Continuous variables were compared using the student t-test. Logistic regression model was used to investigate the effect of various risk factors on stroke subtypes. A value of $p<0.05$ was considered significant. **Results:** One hundred and thirty one stroke patients were included in the study, out of which 93 were ischemic and 38 were haemorrhagic stroke patients. Forty-seven healthy asymptomatic individuals were included as controls Plasma TNF-α levels ($p<0.001$, r=0.503, CI: 18.197–1672.950) were significantly elevated in stroke patients as compared to controls, along with advancing age ($p=0.002$, r=0.310, CI: 1.025–1.110) and history of hypertension ($p=0.002$, r=0.265, CI: 1.746-12.511). Males were found to be at a higher risk of developing stroke. Furthermore, history of hypertension ($p=0.019$, r= -0.294, CI: 0.134–1.500) and detectable TNF- levels ($p=0.002$, r=0.319, CI: 2.106-23.725) were found to be significantly different between ischemic and haemorrhagic stroke patients. **Conclusion:** TNF-α level differed highly significantly between stroke and controls, and also between ischemic and haemorrhagic stroke subtypes.

Keywords: TNF-α, stroke, cytokines, inflammation, stroke, infarct, haemorrhage

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INTRODUCTION

There are two pathological varieties of stroke. These are ischemic and haemorrhagic, of which the ischemic variety is more prevalent.[1] The role of inflammation in stroke pathophysiology[2] is increasingly coming to light. By mediating the pathogenesis of atherosclerosis,[3] inflammation contributes to the occurrence of stroke.

After the cardiovascular event occurs, the ensuing neuronal hypoxia triggers the release of cytokines (including tumour necrosis factor-alpha (TNF-alpha) and Interleukin-10 (IL-10)) from neurons and glial cells. Thus cytokines have been coined as biomarker candidates for stroke. Some studies have reported an association of pro-inflammatory cytokines with risk of acute stroke[4,5] as well with poor prognosis of stroke.[6] However, there is limited data on cytokines levels in stroke subtypes.

The aim of this study was to compare pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 levels in acute ischemic and haemorrhagic stroke patients and investigate their possible association with stroke severity at admission.

MATERIAL AND METHODS

Ethical approval was obtained from the institutional review boards of two public sector hospitals, Shaikh Zayed Hospital Lahore and Services Hospital Lahore, Pakistan. Stroke patients who were admitted to these two hospitals within 24 hours after the onset of stroke symptoms were consecutively asked to participate in this study from June 2011 to December 2011. Informed consent was taken from all the participants or their relatives. All included participants were diagnosed as having stroke (ischemic or haemorrhagic) based on clinical history, physical examination and computed tomography (CT) brain scan by the same physician according to National Institute for Health and Clinical Excellence (NICE) criteria.[7] Transient Ischemic Attacks (TIA) were excluded. A total number of 131 stroke patients were included out of which 93 were ischemic and 38 were haemorrhagic stroke patients. Blood samples were also taken from 47 gender-matched, non-stroke individuals, from the same ethnic background, who served as control group. All blood samples were collected in vacutainers containing ethylenediaminetetra acetic acid (EDTA) within 24 hours of stroke symptoms onset. Plasma was

separated after centrifugation at 3000 rpm for 10 minutes and stored at –20°C until further analysis.

Plasma TNF-α levels and IL-10 were estimated using standard automated procedures with commercial enzyme-linked immune-sorbent assay (ELISA) kits (Diaclon, France), performed in the same laboratory, by the same technician. Sample handling and temperature conditions were strictly monitored for all procedures according to manufacturer's protocol. Cytokines TNF- and IL-10 were undetectable in more than 50% of the samples in at least one of the groups; hence the variable was dichotomized as detectable (yes/no). These variables along with other categorical variables were compared using chi square test. Continuous variables were compared using the student t-test. Continuous and categorical data correlations were assessed by Pearson's and Phi coefficient correlations respectively. Logistic regression model was used to investigate the effect of various risk factors on stroke subtypes. A value of $p<0.05$ was considered significant.

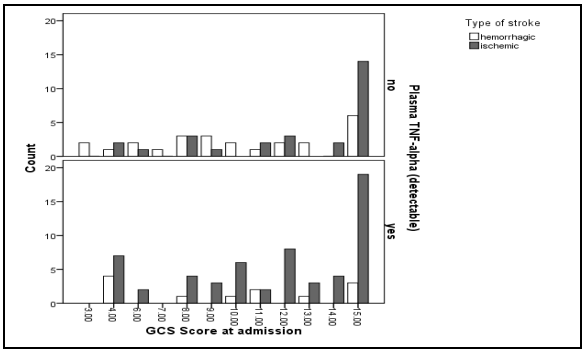
RESULTS

A total of 131 stroke cases and 47 controls were included in this study. Detectable Plasma TNF-α and IL-10 levels were found significantly more in stroke patients than controls, <0.001 and 0.046 respectively. Stroke subtype data showed significant raised detectable plasma TNF-α level in ischemic stroke patients (Table-1 and 2).

A logistic regression analysis was conducted to predict presence of stroke in our sample using age, gender, detectable plasma TNF-α and IL-10 levels, hypertension, diabetes mellitus and smoking status as predictors. A test of the full model against a constant only model was statistically significant, indicating that the predictors as a set reliably distinguished between presence and absence of stroke (chi square=95.948, $p<0.001$ with df=7).

Nagelkerke's R^2 of 0.608 indicated a moderately strong relationship between prediction and grouping. Prediction success overall was 84.8% (90.1% stroke present, 70.2% stroke absent). As shown in table 3, the Wald criterion demonstrated that age, gender, detectable plasma TNF-α levels and hypertension made significant contribution to prediction (p=0.002, 0.046, <0.001 and 0.002 respectively). Detectable IL-10 levels, smoking and diabetes mellitus were not significant predictors. Lastly, analysis indicates that presence of detectable levels of TNF-α in plasma raised the risk of having a stroke by a factor of 174. Similarly age (per year) and hypertension (per mmHg) were found to increase stroke risk by factors of 1.065 and 4 respectively.

We ran a similar model to predict stroke subtype (i.e., ischemic vs haemorrhagic) for the same sample using the same risk factors as in the analysis above. The model was similar in its reliability (chi square =27.264, $p<0.001$ with df =7, Nagelkerke's R^2 of 0.268, prediction success overall was 74.8%). Wald criterion showed only detectable TNF-α plasma levels and presence of hypertension to be significant contributors of prediction (p=0.002 and 0.019 respectively). Finally we found that stroke patients with raised TNF-α were seven times more likely to be suffering from ischemic stroke rather than haemorrhagic stroke.



Graph-A: Stroke subtype data presented as a function of plasma TNF-α detectable level and GCS score. Note the congruence of higher presence of detectable TNF-α level with better GCS scores in ischemic patients. This may indicate the 'protective' function of the cytokine. (GCS Score: Glasgow Coma Scale Score

Table-1: Descriptive and comparative statistical results of data stratified by case-controls group

Variable	Stroke patients (n=131)	Control (n=47)	*p*-value	Correlation r (*p* value)
Mean values (SD)*				
Age	59.1 (14.33)	49.17 (10.77)	<0.001†	0.310 (<0.001†)
WBC count‡	8.13 (2.23)	9.4 (3.43)	0.066	n/a
No. (%)§				
Males	73 (55.7)	39 (83)	0.067	n/a
History of hypertension	81 (61.8)	15 (31.9)	<0.001†	0.265 (<0.001†)
History of heart disease	30 (22.9)	6 (12.8)	0.138	n/a
History of diabetes mellitus	46 (35.1)	9 (19.1)	0.042†	0.152 (0.042†)
Smokers	26 (19.8)	18 (38.3)	0.012†	-0.189 (0.012†)
Dichotomized values (% detectable of total)§				
Detectable Plasma TNF-α levels	77 (58.8)	1 (2.1)	<0.001†	0.503 (<0.001†)
Detectable Plasma IL-10 levels	90 (68.7)	24 (51.1)	0.046†	0.162 (0.046†)

**t*-test used to compare mean values within groups, SD: Standard Deviation, †Significant at $p<0.05$, ‡cells x 10^9 cells per litter, §Chi square test used

Table-2: Descriptive and comparative results of data among stroke subtypes: Ischemic Vs haemorrhagic

	Ischemic stroke	Haemorrhagic stroke		
Variable	n=93	n=38	***p*-value**	**Correlation r (*p*-value)**
Mean values (SD)[*]				
Age	59.89 (14.31)	60.22 (10.22)	0.884	n/a
WBC count[†]	8.18 (2.34)	7.99 (2)	0.770	n/a
GCS score[‡]	11.63 (3.71)	9.86 (4.08)	0.021[§]	0.208 (0.021[§])
No. (%)[d]				
Males	54 (58.1)	19 (50)	0.399	n/a
History of hypertension	49 (52.7)	32 (84.2)	0.001[§]	-0.294 (0.001)
History of heart disease	20 (21.51)	10 (26.3)	<0.552	n/a
History of diabetes mellitus	30 (32.3)	16 (42.1)	0.284	n/a
Smokers	21 (22.6)	5 (13.2)	0.220	n/a
Dichotomized values (% detectable of total)[‖]				
Detectable Plasma TNF-α levels	64 (68.8)	13 (34.2)	<0.001[§]	0.319(<0.001[§])
Detectable Plasma IL-10 levels	67 (72)	23 (60.5)	0.197	n/a

*_t_-test used to compare mean values within groups, SD: Standard Deviation, [†]cells x 10^9 cells per litter, [‡]Glasgow coma scale score, [§]Significant at $p<0.05$, Chi square test was used

Table-3: Logistic regression analysis of stroke Vs controls

Variable	***p*-value**	**Wald**	***p* (95%CI*)**
Age	0.064	9.878	0.002[†] (1.025–1.110)
Gender	-1.174	3.968	0.046[†] (0.097–0.981)
Detectable Plasma TNF-α levels	5.162	20.030	<0.001[†] (18.197–1672.950)
Detectable Plasma IL-10 levels	-0.693	1.931	0.165 (0.188–1.329)
Hypertension	1.542	9.420	0.002[†] (1.746–12.511)
Diabetes Mellitus	-0.222	0.134	0.714 (0.245–2.624)
Smoking	-0.692	1.446	0.229 (0.162–1.546)

*95% Confidence Interval, [†]$p<0.05$

Table-4: Logistic regression analysis of ischemic Vs haemorrhagic stroke

Variable	***p*-value**	**Wald**	**p (95%CI*)**
Age	0.012	0.525	0.469 (0.980 – 1.045)
Gender	-0.067	0.020	0.887 (0.370 – 2.360)
Detectable Plasma TNF-α levels	1.956	10.021	0.002[†] (2.106 – 23.725)
Detectable Plasma IL-10 levels	-0.803	1.695	0.193 (0.134 – 1.500)
Hypertension	-1.250	5.510	0.019[†] (0.101 – 0.814)
Diabetes Mellitus	-0.690	2.180	0.140 (0.201 – 1.253)
Smoking	0.474	0.541	0.462 (0.454 – 5.679)

*95% Confidence Interval, [†]$p<0.05$

DISCUSSION

Cytokines play an important role in a variety of physiological and pathological processes. Although they are implicated in stroke, yet data is still inconsistent about their exact causal relationship with stroke. The precise role that cytokines such as TNF-α and IL-10 play, especially in stroke ischemic animal models, is inconclusive.[8] Furthermore, there are few human studies on the subject. Most studies found on the subject are of case-control design, with very few looking at stroke subtypes.

Our study compared TNF-α and IL-10 in cases and controls as well as stroke subtypes (ischemic and haemorrhagic stroke). We found elevated plasma levels of TNF-α in stroke as compared to controls, and also in ischemic as compared to haemorrhagic stroke ($p<0.001$, 0.002 respectively). TNF-α and ischemic stroke linkage is somewhat controversial, with studies describing both an association[9-11] and a lack of the same.[12,13] There is also the question of the exact nature of the physiological role of TNF-α in the stroke setting i.e. whether it is overall neuro-protective or detrimental.[14,15]. Immunological reaction of CNS tissue to ischemia is likely to affect circulating levels of immune markers. We hypothesize that stroke triggers a general inflammatory response, as reflected by the increased TNF-α levels in peripheral blood in our stroke cases. TNF-α, through its interaction with the blood-brain-barrier, may in turn affect progression of stroke.[15] This is scarcity of data on the role of TNF-α in haemorrhagic stroke in humans, even though it has been studied in the animal models.[16,17]

We found IL-10 levels to be significantly higher in cases as compared to controls (p=0.031), but this significance was lost after adjusting for age, gender and other risk factors (p=0.165) in the regression model. Literature reports raised IL-10 post-acute stroke,[18–21] though the question as to why a so-called anti-inflammatory cytokine would rise during an inflammatory 'storm' led by TNF- α remains to be answered.

Interestingly we found a significant positive correlation between TNF and IL-10 plasma levels in the first 24 hours of stroke symptoms onset. IL-10 is reported to regulate many aspects of immune system including TNF-α. We postulate a pro-inflammatory role of IL-10 directly and indirectly (by enhancing TNF-α levels) at least during the earlier hours of stroke onset. This 'pro-inflammatory' rise in IL-10 has been seen to peak after several days[20], at which time its anti-inflammatory effects seem to become more dominant.

One of the limitations of our study was the cytokine ELISA-based measurement. Secondly, serial cytokine estimation at different time points would also have been more useful in elucidating the exact milieu of these inflammatory markers during stroke progression. Future studies can look at genetic marker analysis of these cytokines for a more detailed account of their significance in clinical stroke.

CONCLUSION

TNF-α level differed highly significantly between stroke and controls, and also between ischemic and hemorrhagic stroke subtypes. With advancing age, male gender, plasma TNF-α levels and hypertension are risk factors for developing stroke. TNF-α seems to be associated with improved GCS scores especially in ischemic stroke.

Conflict of Interest: None

Authors' contributions: FAB: Conception of the idea of this research along with statistical testing, data interpretation and drawing conclusions. TAS: Statistical testing, data interpretation and critical review of manuscript. AB: Diagnosing of stroke patients, timed samples and critical review of the manuscript. FG: Conducting ELISA and data interpretation.

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