

# Oxidative Stress and Post-Ischemic Inflammatory Response in Ischemic Stroke Complicated with Diabetes Mellitus Type 2

Gohar Tsakanova<sup>1</sup>, Elina Arakelova<sup>1</sup>, Armine Soghoyan<sup>2</sup>, Violetta Ayvazyan<sup>1</sup>

<sup>1</sup>Institute of Molecular Biology NAS RA, Armenia

<sup>2</sup>“Surb Grigor Lusavorich” Medical Center CJSC, Armenia

Email: [tsakanova@yahoo.com](mailto:tsakanova@yahoo.com)

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## Abstract

Oxidative stress and post-ischemic inflammatory response are the key pathogenic mechanisms of the neuronal injury caused by ischemic stroke (IS). On the other hand, Diabetes is a major risk factor for the development of stroke, increasing the susceptibility to atherosclerosis and the prevalence of atherogenic risk factors, including hypertension, obesity, and abnormal blood lipids. The aim of current study was to analyze the functional activity of oxidant-antioxidant system and post-ischemic inflammatory response in IS patients complicated and noncomplicated with diabetes mellitus type 2 (DM2). ELISA, photochemiluminescent and spectrophotometric methods were used to analyze the serum samples of IS patients complicated and noncomplicated with DM2, DM2 patients, as well as healthy subjects. The results obtained suggest that IS complicated with diabetes is characterized by higher intensity of the lipid peroxidation process as compared to IS noncomplicated with diabetes that, probably, is one of the determining factors responsible for more severe clinical course of IS patients complicated with DM2 compared to those noncomplicated with DM2. It is also shown that mechanisms of the compensatory response to oxidative stress on the level of antioxidants in IS patients complicated with diabetes differ from those detected in IS noncomplicated with diabetes. A significant increase of the levels of the five analyzed cytokines in both groups of IS patients were detected. Based on the results obtained we suggest that metabolic, molecular, and cellular level alterations are typical for long-term DM2 impair compensatory mechanisms protecting the body from oxidative stress, and that in IS complicated with DM2 the systemic inflammatory reactions and oxidative stress are more intense than in case of IS noncomplicated with DM2.

## Keywords

Oxidative Stress, Postischemic Inflammatory Response, Cytokines, Ischemic Stroke, Diabetes Mellitus

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## 1. Introduction

Stroke is the third leading cause of death and disability worldwide after heart disease and cancer and is the major

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cause of morbidity, particularly in the middle aged and elderly population [1]. On the other hand, diabetes is a major risk factor for the development of stroke, increasing the prevalence of stroke by 20%. Moreover, the mortality rate of stroke is 2.8 - 3.8 times higher in diabetic stroke patients compared to nondiabetic stroke patients [2].

Oxidative stress (OS) and postischemic inflammatory response are the key pathogenic factors leading to uncontrolled cell damage and death, which badly influences stroke progression and outcome [3] [4]. The molecular mechanisms involved in the development of these processes are not clear yet, which is limiting the identification of therapeutic targets for ischemic stroke (IS). The majority of data in this field were obtained in animal models of stroke, which not adequately reflect the pathogenesis of stroke in humans. The important indicators of the development of OS are the elevated levels of lipid peroxidation products and reduced antioxidant capacity of the organism, and the major components of the inflammatory response are cytokines [3] [4].

The present study aimed to reveal the molecular mechanisms responsible for the development of OS and inflammatory reactions in human IS on the systemic level and to identify the molecular components involved in the above mentioned processes. To assess the peculiarities in the development of systemic OS we measured the blood levels of the oxidized derivatives of lipids. To evaluate the functional state of antioxidant system we determined the total capacity of low-molecular non-enzymatic water-soluble antioxidants (TAC) and ferroxidase activity of ceruloplasmin (FAC) in the blood of studied groups. Then, to study the inflammatory reactions we assessed the state of cytokine network in acute ischemic stroke and comparison of functional activities of proinflammatory and chemotactic cytokines, including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MCP-1 and CXCL1, in IS complicated and noncomplicated with DM2 in postischemic inflammatory response progression.

## 2. Materials and Methods

### 2.1. Study Population

In total 90 IS patients complicated with DM2 (mean age  $\pm$  SD: 65  $\pm$  10 years, females/males: 39/51), 120 IS patients noncomplicated with DM2 (mean age  $\pm$  SD: 65  $\pm$  13 years, females/males: 66/54), 110 patients with DM2 (mean age  $\pm$  SD: 59  $\pm$  8 years, females/males: 63/47) and 116 healthy subjects (mean age  $\pm$  SD: 60  $\pm$  12 years, females/males: 69/47) were enrolled in this study (**Table 1**). All subjects were unrelated Caucasians of Armenian ancestry. All IS patients were recruited among those, whose stroke occurred within the prior 24 hours (before any medication was applied), who were consecutively admitted to the Medical Centers of the Ministry of Health of RA. Diagnosis of IS was based on clinical history and neurological examination and was confirmed by brain computer tomography (CT) imaging and standard laboratory analyses. Patients with signs of brain trauma, cerebral hemorrhage or tumors were excluded from the study group. Stroke subtype was assessed according to definitions of TOAST [5].

Patients with large vessel atherothromboembolic stroke (n = 171) and cardioembolic stroke (n = 39) were selected for this study; those with lacunar stroke syndromes were excluded from the study group. Severity of neurological deficit was defined using the National Institutes of Health Stroke Scale (NIHSS). In the present study, patients with a moderate to severe impairment (average NIHSS score 17) were involved. Healthy subjects (controls) were volunteers from the institutes of NAS RA reported no personal or family history of IS, myocardial infarction, and any other cerebrovascular or cardiovascular disease. They had no serious medical disorder or treatment during the past 12 months. Exclusion criteria for all subjects included chronic inflammation or/and infectious diseases, present or past history of metabolic (diabetes mellitus, etc), neuropsychiatric, immune system and oncological disorders, myocardial infarction or any other serious medical conditions. All subjects or their

**Table 1.** Statistical characteristics of investigated groups.

Study Groups	IS complicated with DM2	IS non complicated with DM2	DM2	HS
n	90	120	110	116
Age (M $\pm$ SD), years	65 $\pm$ 10	65 $\pm$ 13	59 $\pm$ 8	60 $\pm$ 12
Female/male	39/51	66/54	63/47	69/47

p values are given in the text.

legal representatives gave their informed consent to participate in the study, which was approved by the Ethical Committee of the Institute of Molecular Biology NAS RA (IRB #00004079).

## 2.2. Methods

Practically fasting blood samples were collected by venipuncture in appropriate tubes and kept on ice for 60 min. After that the coagulated blood was centrifuged at 3000 g for 15 min at 4°C to separate serum from blood corpuscles. The obtained serum samples were stored in aliquots at -30°C and thawed immediately prior to use.

The contents of lipid hydroperoxides and the FAC in the blood serum samples were determined by spectrophotometric assays published earlier [6] [7]. The TAC was determined by photochemiluminescent analysis and the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MCP-1, CXCL1 were measured by ELISA using commercially available kits (Human IL-1 $\beta$ /IL-1F2, Human IL-6 Immunoassay, Human TNF-alpha, Human CCL2/MCP-1, Human CXCL1/GRO $\alpha$  Immunoassays, Quantikine ELISA kits; R & D Systems Europe Ltd; Analytik Jena AG, Germany) according to manufacturers' instructions.

Data statistics was performed by Mann-Whitney's U-test using the "Graphpad Prism" (GraphPad Software Inc., USA) software package. Values of  $p < 0.05$  were accepted as statistically significant.

## 3. Results

The results obtained for the peculiarities in the development of systemic OS showed significantly increased levels of lipid hydroperoxides in the serum of all three patients groups as compared to the group of HS (**Table 2**). Thus, in IS patients complicated and noncomplicated with DM2 the contents of lipid hydroperoxides were 3 and 4 times significantly higher than in HS ( $p < 0.0001$ ). The elevated levels of lipid hydroperoxides in the blood of IS patients complicated and noncomplicated with DM2 indicate the intensification of lipid peroxidation processes induced by IS-associated OS [8]. The same is observed in DM2 patients, although the mechanisms and factors underlying the development of OS in DM2 differ from those found in IS [9]. This, however, explains the fact that the levels of lipid hydroperoxides are 1.3 times significantly higher ( $p < 0.05$ ) in IS patients complicated with DM2 rather than in IS patients noncomplicated with DM2 and, probably, is one of the factors responsible for more severe course of diabetic IS [10] [11].

The results obtained for the functional state of antioxidant system (**Table 2**) showed that the TAC in the blood serum of IS patients noncomplicated with DM2 is 1.4 times significantly higher than in HS and 1.3 and 1.2 times significantly higher than in IS patients complicated with DM2 and DM2 patients, respectively ( $p < 0.0001$ ). However, we did not find any significant differences between the TAC in the blood serum of diabetic IS and DM2 compared to HS ( $p > 0.05$ ).

Concerning the FAC significant differences were found only in the blood serum of IS patients complicated with DM2 (**Table 2**). Thus, according to the results obtained the FAC was 1.3 times significantly higher in the blood serum of IS patients complicated with DM2 compared to HS, as well as IS patients noncomplicated with DM2 and DM2 patients ( $p < 0.0001$ ). In contrast, no significant differences were found between the other groups of the study and HS ( $p > 0.05$ ). Recently it was also shown that the activation of ceruloplasmin can be induced by hypoxia which leads to increased production of superoxide, OS and inflammation [12].

The results obtained in the study of cytokine system showed that the levels of all 5 cytokines measured (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MCP-1 and CXCL1) were higher in diseased groups compared to HS (**Table 3**). Thus, according to the data obtained the levels of IL-1 $\beta$  in the blood serum of IS patients complicated and noncomplicated with DM2 were 8.9 and 5.65 times significantly increased compared to HS ( $p < 0.05$ ), while there were not found any significant differences in the levels of IL-1 $\beta$  in the blood serum of DM2 patients ( $M \pm SD$ :  $6.0 \pm 1.1$  pg/ml) and HS did not differ from the same option in HS ( $M \pm SD$ :  $5.1 \pm 0.8$  pg/ml). Moreover, the levels of IL-1 $\beta$  in the blood serum of IS patients complicated with DM2 were found to be 1.6 times significantly higher than in IS patients noncomplicated with DM2 ( $p < 0.05$ ).

With regard to IL-6, its levels were 3 and 2 times significantly higher in the blood serum of IS patients complicated and noncomplicated with DM2, respectively, compared to HS ( $p < 0.05$ ), while the levels of IL-6 in DM2 patients ( $M \pm SD$ :  $19.2 \pm 3.8$  pg/ml) did not significantly differ from those of HS ( $M \pm SD$ :  $18.4 \pm 3.1$  pg/ml). Though the levels of IL-6 were found to be 1.51 times significantly higher in IS patients complicated with DM2 compared to IS patients noncomplicated with DM2 ( $p < 0.05$ )

According to the results obtained for TNF- $\alpha$ , its levels were 5.2 and 3.7 times significantly increased in IS

**Table 2.** Indicators of OS and the functional activity of antioxidant system (M±SD) in the blood serum of IS patients complicated and noncomplicated with DM2, DM2 patients and HS.

Study Groups	Content of lipid hydroperoxides, A480	TAC, mmol/L	FAC, Umol/L/min
IS complicated with DM2	0.12 ± 0.04	2.8 ± 0.9	574 ± 129
IS noncomplicated with DM2	0.09 ± 0.03	3.4 ± 1.1	475 ± 107
DM2	0.09 ± 0.03	2.6 ± 0.8	483 ± 111
HS	0.03 ± 0.01	2.5 ± 0.8	429 ± 94

p values are given in the text.

**Table 3.** Concentrations (pg/ml) of cytokines (M±SD) in blood serum of IS patients complicated and noncomplicated with DM2, DM2 patients and HS.

Study Groups	[IL-1β]	[IL-6]	[TNF-α]	[MCP-1]	[CXCL1]
IS complicated with DM2	45.4 ± 9.1	55.1 ± 10.8	69.00 ± 13.7	855 ± 140.4	180 ± 31.6
IS noncomplicated with DM2	28.8 ± 4.8	36.5 ± 6.7	50.00 ± 9.6	600 ± 101.7	120 ± 20.7
DM2	6.0 ± 1.1	19.2 ± 3.8	15.00 ± 2.9	280 ± 49.3	78 ± 13.2
HS	5.1 ± 0.8	18.4 ± 3.1	13.37 ± 2.5	254 ± 46.2	74 ± 12.9

p values are given in the text.

patients both complicated and noncomplicated with DM2, respectively, compared to HS ( $p < 0.05$ ). However, we again did not find any significant differences between its levels in DM2 patients ( $M \pm SD$ : 15.00 ± 2.9 pg/ml) and HS ( $M \pm SD$ : 13.37 ± 2.5 pg/ml), but we found significantly high levels (1.38 times) of TNF-α between IS patients complicated and noncomplicated with DM2 ( $p < 0.05$ ).

The data obtained for MCP-1 showed significantly high levels ( $p < 0.05$ ) in IS complicated and noncomplicated with DM2 (3.4 and 2.4 times, respectively), and no difference between the DM2 ( $M \pm SD$ : 280 ± 49.3 pg/ml) and HS ( $M \pm SD$ : 254 ± 46.2 pg/ml). However, the levels of MCP-1 were 1.43 times higher in IS complicated with DM2 than in IS noncomplicated with DM2 ( $p < 0.05$ ).

Finally, the levels of CXCL1 were 2.43 and 1.62 times significantly increased in IS patients complicated and noncomplicated with DM2 ( $p < 0.05$ ), while they did not differ in DM2 patients ( $M \pm SD$ : 180 ± 31.6 pg/ml) and HS ( $M \pm SD$ : 120 ± 20.7 pg/ml). Moreover, the levels of CXCL1 were 1.5 times significantly increased in IS complicated with DM2 than in IS noncomplicated with DM2 ( $p < 0.05$ ).

## 4. Conclusion

Based on the results obtained we suggest that metabolic, molecular, and cellular level alterations are typical for long-term DM2 impair compensatory mechanisms protecting the body from OS, and that in IS complicated with DM2 the systemic inflammatory reactions and OS are more intense than in case of IS noncomplicated with DM2.

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