ORIGINAL ARTICLE

The Study of Paraoxonase 2, Lipid Profile, and Total Oxidative Stress in Ischemic Stroke

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ABSTRACT

Background: Paraoxonase2 **(**PON2) is an intracellular protein and widely present in many tissues such as endothelial cell and heart. It plays a major role in prevention of low-density lipoprotein (LDL) oxidation and reverses mildly oxidized LDL. PON2 plays an important role in antioxidation, antiatherosclerotic, and anti-inflammatory function. The oxidative stress leading to ischemic cell death involves the formation of reactive oxygen species/reactive nitrogen species through multiple injury mechanism. **Aim:** The aim of our study was to assess and correlate monocytic PON2 activity, lipid profile, and total oxidative stress in patients with ischemic stroke. **Materials and Methods**: The study population was included 50 ischemic stroke patients as cases and 50 healthy controls. The monocytic PON2 was measured spectrophotometrically using dihydrocoumarin as substrate. Serum lipid profile was estimated by established biochemical methods. **Results:** The monocytic PON2 activity showed significant decrease in case ($P < 0.001$) while total oxidative stress showed significantly increases in cases ($P < 0.001$). Serum total cholesterol is significantly increases in cases (P value <0.001) and serum HDL showed significant decrease in case ($P < 0.005$). Monocytic PON2 activity is significantly correlated with total oxidative stress (TOS) $P = 0.000$). Multivariate logistic regression analysis used for prediction of risk of ischemic stroke contributed by various risk factor. The basic Model I shows significant P value and Naglekerke R^2 value is 0.479. When PON2 activity is added to the basic model in the Model II, Naglekerke R^2 value changes from 0.479 to 0.522, it also shows independent association of PON2 activity towards ischemic stroke. In linear regression analysis, significant positive correlation observed between PON2 and Ischemic stroke. PON2 lactonase activity was negatively correlated with total oxidative stress. **Conclusion:** Our study strongly suggests that the estimation monocytic PON2 and total oxidative stress give valuable information for prediction of risk of ischemic stroke due to cerebrovascular thromboembolism and may consider as risk factors.

KEY WORDS: Paraoxonase 2, total oxidative stress, lipid profile, ischemic stroke.

Introduction

Ischemic stroke is the cessation of blood flow in arteries lasts for more than a few minutes' which leads to infarction or death of brain tissues. The arteries are blocked due to blood clot or an atheromatous plaque. Atherosclerosis is the most common pathological feature of vascular obstruction resulting in thrombotic stroke.[1] The arterial occlusion leads to the neuronal cell death. The cascade after cerebral ischemia involves the variety of complex pathophysiological mechanisms in which chronic inflammation plays an important role. The resulting inflammation leads to tissue injury which then activates different pro-inflammatory mediators.[2] The formation of ox-low-density lipoprotein (LDL) plays a most important role in the pathogenesis of atherosclerosis and is an initiating event of fatty streak or plaque.[3] The activated macrophages also produce reactive oxygen species that aggravate LDL oxidation and produce growth factor which leads to smooth muscle cell proliferation and promotes cell death or apoptosis.[2] The mechanism of cerebral ischemia is shown in Figure 1.

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species or free radicals and antioxidant.

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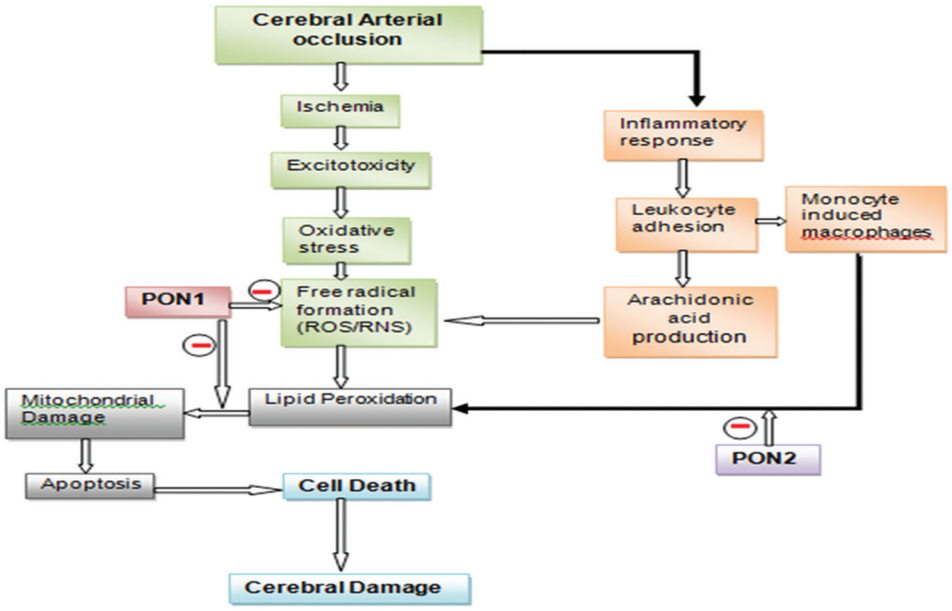


Figure 1: Flowchart of mechanism of cerebral ischemia

The oxidative stress leading to ischemic cell death involves the formation of free radicals such as reactive oxygen species/reactive nitrogen species through multiple injury mechanism. Along with the production of oxidants, there is excessive consumption of antioxidants (such as Vit. A, E, C, and uric acid) by free radicals during ischemia.[4-7]

Paraoxonase 2 (PON2)

PON2 is a member of the PON gene family. It is consists of three proteins such as PON1, PON2, and PON3. PON2 is an intracellular membrane-associated protein. It is present mainly in vascular cells such as endothelial cell and many tissues such as lungs, liver, kidney, and heart.[8] PON2 possesses only lactonase activity. It plays a major role in prevention of ox-LDL formation, increases cholesterol efflux, and decreases the size of atherosclerotic plaque.[9] PON2 plays an important role in antioxidation, antiatherosclerotic, and anti-inflammatory function.

The aim of our study was to estimate and correlate Paraoxonase-2, lipid profile and total oxidative stress in ischaemic stroke patients.

Materials and Methods

Study design – This was a hospital-based cross-sectional case–control study

Cases – Fifty ischemic stroke diagnosed patients
Control – Age- and sex-matched 50 healthy subjects.

Confirmation of diagnosis were done by clinicians which includes the clinical signs and symptoms such numbness (paresthesia) or weakness (paresis) in the face, arm or leg, usually on one side of the body (hemiparesis); difficulty in speaking (expressive aphasia); slurred speech (dysarthria) and radiological findings such as CT scan. Subjects with previous history of IHD, intracranial haemorrhage, diabetes mellitus and chronic renal or hepatic diseases were excluded. Written valid informed consent was obtained from all subjects. The study was approved by the Institutional Ethical Committee. Sample – fasting blood sample was collected in heparin for monocytes activity and in plain tube for lipid profile and total oxidative stress and was analyzed within a few hours of collection.

Serum Lipid

Serum lipid profile such as total cholesterol, high-density lipoprotein (HDL), and triglycerides (TGs) was estimated on random assess clinical chemistry autoanalyzer.

Serum cholesterol and HDL cholesterol (HDL-C) were estimated by Cholesterol Oxidase Phenol 4-aminoantipyrine Peroxidase (CHOD-PAP) method

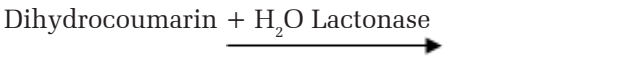
Serum TGs were measured by the enzymatic GPO-PAP method

LDL cholesterol (LDL-C) was calculated by Friedewald formula:

LDL-C= Total serum cholesterol – (HDL-C + TG/5) mg/dl.

Paraoxonase 2 lactonase assay (monocytic)[10]

Principle: Monocyte lactonase catalyzes hydrolysis of dihydrocoumarin (DHC) to form 3-(2-hydroxyphenyl) propanoic acid.



Reagents

1. HiSep LSM media (Monocytes separating media by HiMedia Laboratories)
2. Isotonic phosphate buffered saline
3. Substrate - Dihydrocoumarin (DHC)

Procedure

1. Make a 1:2 dilution of the heparinised whole blood with isotonic phosphate buffered saline.
2. Transfer 2.5 ml of HiSep LSM to a 15 ml clean centrifuge tube and overlay with 7.5 ml diluted blood.
3. Centrifuge at 3000 rpm at room temperature for 15-30 minutes. Aspirate most of the plasma and platelet containing supernatant along with HiSep media and discard.
4. Again aspirate and collect the white layer of monocytes using clean glass pipette and transfer in centrifuge tube.
5. Add equal volume of isotonic phosphate buffered saline to monocytes layer, mix and centrifuge for 15 minutes.
6. Repeat the previous step 2 times again. This washing removes whole of the HiSep media.
7. Resuspend monocytes in clean plain bulb.
8. The monocytes were lysed by using chilled distilled water and 26 gauge needle. Proteins in lysed monocytes were estimated by Lowry's method.

Lysed monocyte protein estimation was performed using Lowry's method.[11] Mononuclear cells are separated from whole blood using monocyte separation media. The assay mixture contains 1.0 mM/L DHC, 1 mM/L $CaCl_2$, and 50 mM/L Tris-HCl buffer, pH 8.0 at 25°C. The reaction was initiated by adding 10 µl lysis sample in 2 ml assay mixture. The increase in rate absorbance of acid formation was recorded spectrophotometrically at 270 nm. The difference in molar extinction coefficient of substrate and product is 1295 $M^{-1}cm^{-1}$ at 270 nm and pH 8.0 at 25°C after correction of non-enzymatic hydrolysis. Non-enzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. Monocytic PON2 lactonase activity was expressed as U/mg protein. One unit of lactonase activity is equal to 1 mM of DHC hydrolyzed per min/ml and then converted into U/mg.

Calculation:

Lactonase activity (U/mg of cell protein) = ΔA /min × 155.212

Total oxidative status (TOS) assay[12]

Principle

Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complexes into ferric ions. The reaction is enhanced by glycerol molecules that are abundantly present in the reaction medium. The ferric ions form a colored complex with xylenol orange in an acidic medium. Therefore, the color intensity measured spectrophotometrically is related to the total number of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equiv./L).

Statistical analysis

Results are presented as mean ± standard deviation.

Student's unpaired *t*-test used for statistical analysis between cases and controls.

The strength of association between two parameters is expressed by the Pearson's correlation coefficient. The logistic regression analysis is used for prediction of risk of ischemic stroke contributed by various risk factors. At each step, variable not in the model is assessed for its contribution to the model reflected by the Nagelkerke R^2 value and *P* value of the model. The two models prepared in the logistic regression for the analysis of data are as follows:

Model I: TC, HDL-C, LDL-C, and TOS (known risk factors).

Model II: All parameters in Model I + PON2 activity.

Odds ratio and 95% confidence intervals are calculated. $P < 0.05$ is considered as statistically significant. All analyses are carried out with the statistical software.

Results

In Table 1, age and gender distribution of cases and control is shown. In Table 2, serum levels of total cholesterol and LDL-C are higher in cases than in controls and are statistically significant. Serum TG and very low-density lipoprotein (VLDL) levels are higher in cases but not significant. However, serum HDL-C levels are decreased significantly in ischemic stroke patients when compared with control group. Monocytic PON2 activity also showed significant decrease in cases (1.496 ± 1.080 U/mg) as compared to control group (2.789 ± 1.984 U/mg). Total oxidative stress showed significant increase in cases (28.71 ± 8.92 µmoles H_2O_2 equiv/L) as compared to control group (20.63 ± 8.71 µmoles H_2O_2 equiv/L). Monocytic PON2 activity is significantly correlated with total oxidative stress (TOS) (Figure 2 correlation coefficient – −0.326, P = 0.000).

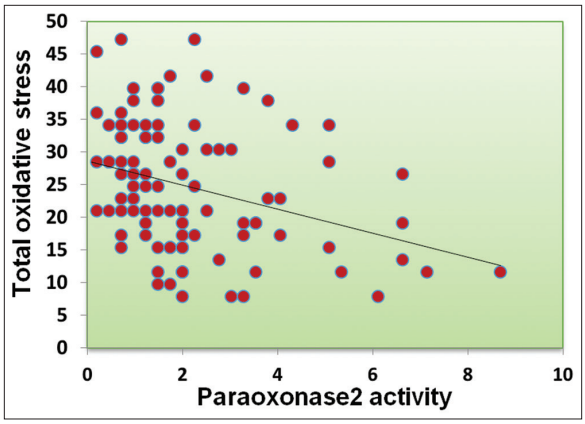


Figure 2: Correlation between total oxidative stress and PON2 activity (r = −0.326, P = 0.001)

Table 1: Clinical parameters of ischemic stroke cases and controls

Parameters	Cases (n=50)	Controls (n=50)	P-value
Age	64.88±8.27	61.66±8.62	NS
Gender (male/female)	21/29	24/26	NS

Logistic regression analysis

Model I: Nagelkerke R^2 =0.479, P=0.000

Parameter	SE	Z	OD (95% CI)	P-value
TC	0.018	−0.522	0.991 (0.957–1.026	0.602
HDL	0.045	3.539	1.150 (1.064–1.243)	0.000
LDL	0.018	−1.012	0.982 (0.947–1.018)	0.312
TOS	0.029	−3.092	0.905 (0.850–0.964)	0.002

LDL: Low-density lipoprotein, HDL: High-density lipoprotein

Model II: Nagelkerke R^2=0.522, P=0.000

Parameter	SE	Z	OR (95% CI)	P-value
TC	0.018	−0.081	0.999 (0.964–1.034)	0.936
HDL	0.046	3.225	1.139 (1.052–1.233)	0.001
LDL	0.018	−1.321	0.976 (0.941–1.012)	0.187
TOS	0.031	−2.699	0.913 (0.854–0.975)	0.007
PON2	0.323	2.061	1.540 (1.021–2.322)	0.039

LDL: Low-density lipoprotein, HDL: High-density lipoprotein, OR: Odds ratio, CI: Confidence interval

Multivariate logistic regression analysis used for prediction of risk of ischemic stroke contributed by various risk factors. The basic Model I shows significant P value and Nagelkerke R^2 value is 0.479. When PON2 activity is added to the basic model in the Model II, Nagelkerke R^2 value changes from 0.479 to 0.522, it also shows independent association of PON2 activity toward ischemic stroke.

In this multivariate model, finding suggests that PON2 and total oxidative stress and also lipid profile are significantly associated with the ischemic stroke and are independent predictors and risk for ischemic stroke.

Table 2: Biochemical parameters of ischemic stroke cases and controls

Parameters	Cases (n=50)	Controls (n=50)	P-value
Total cholesterol (mg/dl)	201.66±38.73	177.94±28.79	<0.001
TG (mg/dl)	174.90±36.94	167.12±36.75	NS
HDL-C (mg/dl)	30.46±7.42	35.48±6.97	<0.005
LDL-C (mg/dl)	136.40±35.88	108.62±30.06	<0.001
VLDL-C (mg/dl)	34.56±7.68	31.84±7.37	NS
Total oxidative stress (TOS) (µmoles H_2O_2 equiv./L)	28.71±8.92	20.63±8.71	<0.001
PON 2 activity (U/mg)	1.496±1.080	2.789±1.984	<0.001

HDL-C: High-density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol, TG: Triglyceride, VLDL-C: Very low-density lipoprotein cholesterol

Discussion

The present case–control study was carried out in Government Medical College and Hospital. A total of 50 diagnosed cases of ischemic stroke admitted were recruited in this study and 50 normal healthy control subjects of matched age and sex were taken for comparison. The aim of present study was to determine the relation of PON2 enzyme activity and total oxidative stress with the ischemic stroke patients. The parameters studied were monocytic PON2 activity, total oxidative stress, and lipid profile.

In this study, serum HDL-C levels were significantly decreased in cases. Serum total cholesterol and LDL-C were significantly higher in ischemic stroke patients.

The present study demonstrated for the 1st time that monocytic PON2 was significantly decreased in cases of ischemic stroke than in control subjects (1.49 ± 1.08 U/mg vs. 2.78 ± 1.984 U/mg, $P < 0.001$).

As PON2 is present in intracellularly in many tissues such as on monocytes, macrophages and has antioxidant and antiatherosclerotic properties,[13] we hypothesized that PON2 lactonase activity expected to decrease in monocytes obtained from cerebrovascular ischemic stroke patients. To the best of our knowledge, it is the first study on PON2 activity in ischemic stroke cases and till date, no literature is available on PON2 activity or concentration. In stroke, oxidative stress is one of the reasons for neuronal damage and it may be possible to reduce this stress by PON2 enzyme by reducing the ox-LDL accumulation in monocytes derived macrophages in carotid atherogenic lesions. The present study revealed significantly lower values of PON2 in cases than controls (1.49 ± 1.08 U/mg vs. 2.78 ± 1.984 U/mg, $P < 0.001$).

For the 1st time, Devarajan *et al*.[14] demonstrated that PON2 deficiency affects mitochondrial function and increases development of atherosclerosis. They showed localization of PON2 to the inner mitochondrial membrane and suggest that deficiency of PON2 leads to dysfunction of mitochondria together with an increase in mitochondrial oxidative stress.

In the study, Ng *et al*.[8] demonstrated for the 1st time that PON2 is able to lower the intracellular oxidative stress of a cell and prevent the cell-mediated oxidation of LDL.

Rosenblat *et al*.,[15] they stated that increased macrophage PON2 expression under oxidative stress may represent a selective cellular response to reduce oxidative stress burden. Another most important study by Fortunato *et al*.[16] who first demonstrated that both mRNA and protein of PON2 are decreased in human advanced carotid plaque.

The decreased monocytic PON2 activity in ischemic stroke suggests that PON2 is an additional factor for ischemic neuronal injury.

The large amount of experimental studies showed the production of oxygen- and nitrogen-derived free radicals (reactive oxygen/nitrogen species, ROS/RNS) leading to oxidative stress which plays an important role in pathophysiology of ischemic brain damage. Relatively human studies on stroke and oxidative stress are few mainly because of the methodological difficulties in measuring free radical production in cerebral tissue.[7,17] Since each different types of oxidant biomolecules or metabolites measurement are not practically possible, we measured total oxidative status in case–control samples. Furthermore, there are very few studies done on total oxidative stress level in ischemic stroke patients.

In our study, total oxidative stress level was significantly higher in ischemic cases in comparison to controls. The total oxidative stress was significantly negatively correlated with PON2.

In one of the study of Aygül *et al*.[18] showed the plasma malondialdehyde, nitric oxide and homocysteine levels were significantly higher in the stroke patients as compared to controls ($P < 0.001$). These data support that free radical mechanisms may play a role in the development of brain injury following ischemic stroke. El Kossi and Zakhary[19] demonstrated the free radical generation and consequent oxidative stress in thrombotic cerebrovascular stroke and determined levels of plasma homocysteine, lipid peroxide, ascorbic acid, superoxide dismutase, and nitric oxide.

However, all the parameters, except TG and VLDL cholesterol, show significant association with ischemic stroke in univariate analysis of logistic regression while in multivariate logistic regression, only PON2 and HDL-C remain significant after adjustment of other risk factors of ischemic stroke. This suggests that PON2 and HDL-C are independent predictor for risk of ischemic stroke.

The present study demonstrates that the ischemic stroke is associated with significant change in TC, HDL-C, monocytic PON2, and TOS. We conclude that decreased PON2 and raised TOS may reflect oxidative stress. The atheroprotective effects of HDL-C may be contributed by PON1 enzyme and also decreased monocytic PON2 may be an additional factor for ischemic neuronal injury in ischemic stroke. The present study is a cross-sectional study which has major and distinctive strengths contemplated to be the first study in ischemic stroke cases determining the effect of PON2 activity. To clarify and demonstrate the role PON2 and TOS in ischemic stroke, further more epidemiological studies are required.

Limitations of Study:

The limitation of our study is small sample size.

Conclusion

From this study, we conclude that PON2 lactonase is reduced in ischemic stroke. The estimation of monocytic PON2 and total oxidative stress gives valuable information for prediction of risk of ischemic stroke. In future, further more studies are required for the assessment and effect of PON2 and TOS in ischemic stroke.

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