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Fibrinogen nitrotyrosination after ischemic stroke impairs thrombolysis and promotes neuronal death

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ABSTRACT

Ischemic stroke is an acute vascular event that compromises neuronal viability, and identification of the pathophysiological mechanisms is critical for its correct management. Ischemia produces increased nitric oxide synthesis to recover blood flow but also induces a free radical burst. Nitric oxide and superoxide anion react to generate peroxynitrite that nitrates tyrosines. We found that fibrinogen nitrotyrosination was detected in plasma after the initiation of ischemic stroke in human patients. Electron microscopy and protein intrinsic fluorescence showed that *in vitro* nitrotyrosination of fibrinogen affected its structure. Thromboelastography showed that initially fibrinogen nitrotyrosination retarded clot formation but later made the clot more resistant to fibrinolysis. This result was independent of any effect on thrombin production. Immunofluorescence analysis of affected human brain areas also showed that both fibrinogen and nitrotyrosinated fibrinogen spread into the brain parenchyma after ischemic stroke. Therefore, we assayed the toxicity of fibrinogen and nitrotyrosinated fibrinogen in a human neuroblastoma cell line. For that purpose we measured the activity of caspase-3, a key enzyme in the apoptotic pathway, and cell survival. We found that nitrotyrosinated fibrinogen induced higher activation of caspase 3. Accordingly, cell survival assays showed a more neurotoxic effect of nitrotyrosinated fibrinogen at all concentrations tested. In summary, nitrotyrosinated fibrinogen would be of pathophysiological interest in ischemic stroke due to both its impact on hemostasis – it impairs thrombolysis, the main target in stroke treatments – and its neurotoxicity that would contribute to the death of the brain tissue surrounding the infarcted area.

1. Introduction

Stroke is a leading cause of death and disability worldwide [1,2]. The effectiveness of ischemic stroke treatment depends on its rapid and accurate diagnosis [3]. The currently available treatment target is clot lysis with recombinant tissue plasminogen activator (rtPA) but carries a certain risk of bleeding and less than 5% of stroke patients receive this treatment due to the very narrow therapeutic window for rtPA (4.5 h after stroke onset) [4].

Ischemic stroke is an acute vascular event that hinders blood supply to the brain and leads to an ischemic process that affects neurons, glial cells and vessels. The tissue surrounding the ischemic core lesion is

Abbreviations: Ab, antibody; BSA, bovine serum albumin; CP, cortical perfusion; FBS, fetal bovine serum; GSH, reduced glutathione; MCA, middle cerebral artery; MG, methylglyoxal; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NTyr, 3-nitrotyrosine; $O_2^{\cdot-}$, superoxide anion; $ONOO^-$, peroxynitrite anion; o.n., overnight; RT, room temperature; rt-PA, recombinant tissue plasminogen activator; SH-SY5Y, human neuroblastoma cells; SIN-1, 3-morpholinosydnonimine hydrochloride; SNP, sodium nitroprusside

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termed "penumbra", a region where neurons are still viable owing to residual blood perfusion [5]. In this scenario, the liberation of nitric oxide (NO), a molecule with pleiotropic effects in the brain [6], is increased to favor vasodilatation and blood supply to the compromised brain region [7], while reperfusion after thrombolysis induces a burst of free radicals such as the superoxide anion ($O_2^{-\cdot}$) [8,9]. NO reacts with O_2^- producing the highly reactive peroxynitrite anion ($ONOO^-$) [10], which, among other harmful effects, irreversibly nitrates proteins [11,12]. This process, known as nitrotyrosination, is a post-translational modification that normally leads to a loss of protein function.

Fibrinogen is one of the most abundant plasma proteins. Its main physiological function is hemostasis as a result of its aggregation to the fibrin polymers that mediate clot formation. Increased levels of circulating fibrinogen have been identified as a stroke risk factor [13] as well as a bad prognosis factor after stroke [14–16]. In the present work we have evaluated the relevance of fibrinogen nitrotyrosination in ischemic stroke, with particular emphasis on hemostasis and cell toxicity.

2. Materials and methods

2.1. Biological material

Human brain sections obtained from autopsies of patients who had an ischemic stroke were provided by the Servei d'Anatomia Patològica (Hospital del Mar, Barcelona) corresponding to 4 patients who were 68 ± 34 years old (2 men and 2 women). Human plasma and anticoagulant free whole blood samples, provided by the Servei de Neurologia (Hospital del Mar), were obtained from patients after ischemic strokes and from controls. Blood extraction was carried out approximately 3 h after ischemic stroke. Controls were 53 ± 4 years old (14 men and 9 women); atherothrombotic stroke patients were 70 ± 2 years old (8 men and 9 women); cardioembolic stroke patients were 78 ± 2 years old (5 men and 9 women); undetermined stroke patients were 72 ± 4 years old (2 men and 7 women); and lacunar stroke patients were 67 ± 4 years old (7 men and 5 women). All procedures were approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques — Universitat Pompeu Fabra. Subjects gave informed consent.

Plasmas from rat were obtained from 3-month old Sprague–Dawley rats. The procedure was approved by the Ethics Committee of the Universitat de Barcelona.

Human neuroblastoma cell line (SH-SY5Y), supplied by ECACC, were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA).

2.2. Clot formation

Anti-coagulant free whole blood samples were treated immediately after extraction with phosphate-buffered saline (PBS; controls), sodium nitroprusside (SNP; a NO donor, Sigma, St. Louis, USA) + H_2O_2 or H_2O_2 (Sigma, St. Louis, USA). They were allowed to clot for 3 h at 37 °C. Clots were removed and embedded in optimal cutting temperature compound (Sakura Finetek) medium and frozen to −80 °C. The clots were analyzed by immunodetection as described below to study the structure of the fibrin network and the presence of nitrotyrosination.

2.3. Immunodetection

Formalin-fixed paraffin-embedded brain sections were cut at 3 μm, deparaffinated at 70 °C for 1 h and washed with decreasing concentrations of ethanol. Antigen retrieval was performed with proteinase K at 40 μg/mL in a 1:1 glycerol and TE buffer solution. Frozen clots were cut at 5 μm in a cryostat. Immunostaining was performed with 1:200 mouse monoclonal anti-3-nitro-tyrosine (NTyr; Cayman Chemical, Michigan, USA) antibody (Ab) or 1:200 rabbit polyclonal anti-human fibrinogen (Dako, California, USA) Ab for 2 h at room temperature (RT) followed by 1:1000 Alexa555-bound anti-mouse or 1:1000 Alexa488-bound anti-rabbit as secondary Abs (Dako, California, USA) overnight (o.n.) at 4 °C. Sections were stained with TO-PRO to identify the nuclei and mounted with Mowiol. Images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Leica).

SH-SY5Y cells (4×10^4 cells/well) were seeded on 1.5% gelatin-coated 12 mm coverslips. Cells were treated for 45 min with PBS (controls), 5 μg/μL fibrinogen (Sigma, St. Louis, USA) or 5 μg/μL nitrofibrinogen prepared by pre-incubating fibrinogen with 100 μM 3-morpholinosydnonimine hydrochloride (SIN-1; Sigma, St. Louis, USA), an $ONOO^-$ donor. They were fixed after 45 min (fibrinogen challenge) and incubated for 2 h at RT with 1:500 rabbit anti-cleaved caspase-3 (Asp175) Ab (Cell Signaling, Beverly, USA) followed by 1:500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab for 1 h at RT. Cells were stained with TO-PRO to identify the nuclei and mounted with Mowiol. Images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Leica).

2.4. Focal cerebral ischemia in rats

Focal cerebral ischemia was produced by transient intraluminal occlusion of the middle cerebral artery (MCA) in rats, as previously reported [17]. Plasma (500 μL) was extracted from the rats after 3, 6, 12, and 24 h of MCA occlusion. The procedure was approved by the Ethics Committee of the Universitat de Barcelona (CEEA-273/09).

2.5. Fibrinogen immunoprecipitation

Human and rat plasma samples (350 μL) were incubated with 5 μg of anti-human fibrinogen polyclonal Ab or to 5 μg of anti-rat fibrinogen polyclonal Ab (Accu-Specs, Westbury, NY, USA). Samples were shaken overnight at 4 °C. Following addition of 20 μg of protein G-sepharose (Sigma, St. Louis, USA), samples were shaken for 2 h at 25 °C, centrifuged at 10,000 rpm for 10 min and washed 3 times with PBS.

2.6. Quantification of nitrotyrosination

Immunoprecipitated fibrinogen was used at a final volume of 100 μL. NTyr was determined spectrophotometrically by the absorbance measurement at 415 nm (pH > 9) [18]. A calibration curve using serial dilutions of free NTyr (Sigma, St. Louis, USA) was used to quantify nitrotyrosination expressed as NTyr μg/μL or mol of NTyr/mol of Fib.

2.7. Fibrinogen conformational state analysis by intrinsic fluorescence measurement

Intrinsic protein fluorescence emission is mainly due to tryptophan residues, which have a wavelength of maximum absorption of 280 nm and an emission peak ranging from 300 to 350 nm. Therefore the shift in protein fluorescence can be used to study the changes in protein conformational states [19]. Freeze-dried human fibrinogen was directly solubilized at 1 mg/mL in TBS (50 mM Tris base, 150 mM sodium chloride; pH 7.4). Fibrinogen was then incubated in the presence of 12 mM $CaCl_2$, with or without 100 μM SIN-1 at 37 °C for 24 h in the dark. Intrinsic fluorescence measurements of these mixtures were determined in a Shimadzu spectrofluorophotometer (RF-5301). Samples were excited at 280 nm and fluorescence emission reading was recorded between 300 and 400 nm.

2.8. Transmission electron microscopy

Samples of 5 μL of fibrinogen (1 mg/mL) incubated in the absence or presence of 100 μM SIN-1 were applied to carbon-coated Formvar grids (Sigma, St. Louis, USA). Samples were stained with 5 μL of 0.5% phosphotungstic acid solution (Sigma, St. Louis, USA). Samples were examined under a JEOL 1200 EX II electron microscope at 80 kV.

2.9. Thromboelastometry studies

Whole blood from control donors was incubated with 10 mM SNP and 50 μM H_2O_2, to induce a superoxide anion production, or only with 50 μM H_2O_2, a control for oxidative stress, at 37 °C for 1 h and evaluated using the ROTEM Thromboelastometry Analyzer (Pentapharm GmbH, Munich, Germany). This technique was performed according to the manufacturer's instructions. TEM studies focused on analysis of the ExTEM and FibTEM tests. Briefly, ExTEM evaluates viscoelastic changes in fibrin polymerization and platelet contractile resistance induced through the activation of the extrinsic coagulation pathway by tissue factor. The FibTEM test is also based on activation of the extrinsic coagulation pathway, but includes cytochalasin that inhibits platelet cytoskeletal contraction. Viscoelastic properties of clots formed with the ExTEM are contributed by polymerizing fibrin and platelets, whereas those formed with the FibTEM are mainly contributed by fibrin. This technique provides information through different parameters. We assessed four variables. The clotting time (CT), defined as the time elapsed from the measurement start until the amplitude of the forming clot reaches 2 mm. The clot formation time (CFT) is the time from the start of clot formation until this clot reaches 20 mm of amplitude. CT and CFT indicate the dynamics of clot formation. The maximum clot firmness (MCF) was evaluated as a measure of clot firmness. The clot amplitude gives information about clot strength and stability, which is largely dependent on fibrinogen and platelets. The maximum lysis (ML) describes the degree of fibrinolysis relative to MCF achieved during the measurement (% clot firmness lost). Time 0 is based on shear. All analyses were carried out for 1 h.

2.10. Coagulation pathway study in samples from stroke patients

Blood samples from stroke patients were collected in tubes with sodium citrate. The intrinsic pathway was activated by adding calcium. Time was measured until clot formation (Partial Thrombin Time; PTT) and expressed in seconds. The extrinsic pathway was activated by adding tissue factor (factor III) to isolated plasma. Time was measured until clot formation (Prothrombin Time; PT) and expressed as the International Normalized Ratio multiplied by 100.

2.11. Cell viability assays

SH-SY5Y cells (10^4 cells/well) were treated with increasing concentrations of fibrinogen or nitro-fibrinogen for 24 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method and expressed as a percentage of control [11].

2.12. Statistics

All data are expressed as mean ± SEM of n independent samples/experiments analyzed in duplicate or triplicate. Statistical analysis was performed with paired Student's *t*-test for two comparisons and one-way ANOVA followed by LSD post-hoc analysis for multiple comparisons. $p < 0.05$ was considered significantly different from the reference value. Correlation analysis was carried out with the Pearson Correlation Test.

3. Results

3.1. Ischemic conditions induce nitro-oxidative stress and fibrinogen nitrotyrosination

Human brain slices obtained from autopsies of stroke patients were analyzed for the presence of nitro-oxidative stress. A diffuse nitrotyrosination staining (Fig. S1A), secondary to peroxynitrite anion ($ONOO^-$) production, was detected in the infarcted area (previously identified post-mortem at the Hospital del Mar Pathology Department). Unaffected areas located in the same brain slice showed no signs of nitrotyrosination. The presence of advanced glycation end-products (AGEs), indicative of radical oxygen species production [20,21], was also detected in infarcted areas but absent in unaffected regions (Fig. S1B). Similarly, human neuroblastoma cells (SH-SY5Y) subjected to oxygen and glucose deprivation (OGD) for 1 h followed by reoxygenation and immersion in glucose-containing medium (mimicking the ischemia–reperfusion process that occurs during stroke) [22], showed higher levels of protein nitrotyrosination (Fig. S1C), and oxidative stress, as revealed by AGEs (Fig. S1D), than control cells. Brain ischemic conditions trigger nitro-oxidative stress and the generation of peroxynitrite [23]. Ischemic stroke also alters the permeability of the blood brain barrier enabling the extravasation of plasmatic proteins into the penumbra area of the infarcted brain parenchyma [24,25] but, is it possible for the peroxynitrite produced during the ischemic conditions of a stroke to spread its harmful effects to plasma proteins? Peroxynitrite is a highly reactive compound and under physiological conditions has a half-life of 1–2 s and an action radius of 100 μm [10]. These properties make it possible after an ischemic process for the peroxynitrite burst to reach the lumen of blood vessels affecting the circulating plasmatic proteins.

We focused our attention on fibrinogen, which is the third most abundant protein in plasma and plays a key role in hemostasis, a critical physiological process in stroke progression. Using an anti-fibrinogen antibody to analyze the brain parenchyma, we found an intense signal corresponding to fibrinogen extravasation that partially colocalized with the nitrotyrosination staining in the parenchyma from an ischemic stroke patient (Fig. 1A).

We then analyzed nitrotyrosination of plasma fibrinogen in both an animal model of brain ischemia, and in stroke patients. Transient occlusion of MCA in rats produced an infarction affecting the ipsilateral cortex and striatum. The mean infarct volume was 315.9 ± 64.4 mm^3 (mean ± SEM), measured as previously described [26]. Plasma samples were extracted from rats before induction of ischemia (0 h) and at 3, 6, 12, and 24 h after MCA occlusion. A large increase in plasmatic nitrotyrosinated fibrinogen (NTyr) was detected with a peak at 3 h (Fig. 1B). Elevated levels of nitrotyrosination were detected up to 24 h after MCA occlusion demonstrating that nitrotyrosination of fibrinogen was maintained at high levels as a direct consequence of brain ischemia.

The presence of nitrotyrosinated fibrinogen was also measured in human plasma samples approximately 3 h after suffering different types of ischemic stroke: atherothrombotic, cardioembolic, undetermined and lacunar. The levels of nitrotyrosinated fibrinogen obtained from patients suffering from any type of ischemic stroke were significantly higher than those detected in healthy controls ($p < 0.01$ for atherothrombotic stroke; $p < 0.05$ for cardioembolic and undetermined strokes; and $p < 0.0005$ for lacunar stroke; Fig. 1C).

Nitrotyrosination of plasma proteins from healthy donors was also evaluated *in vitro*. A wide range of proteins with different molecular sizes can be extensively nitrotyrosinated by the peroxynitrite donor SIN-1 in a dose-dependent manner (Fig. S2A). The nitrotyrosination was prevented by the natural antioxidant reduced glutathione (GSH) (Fig. S2B).

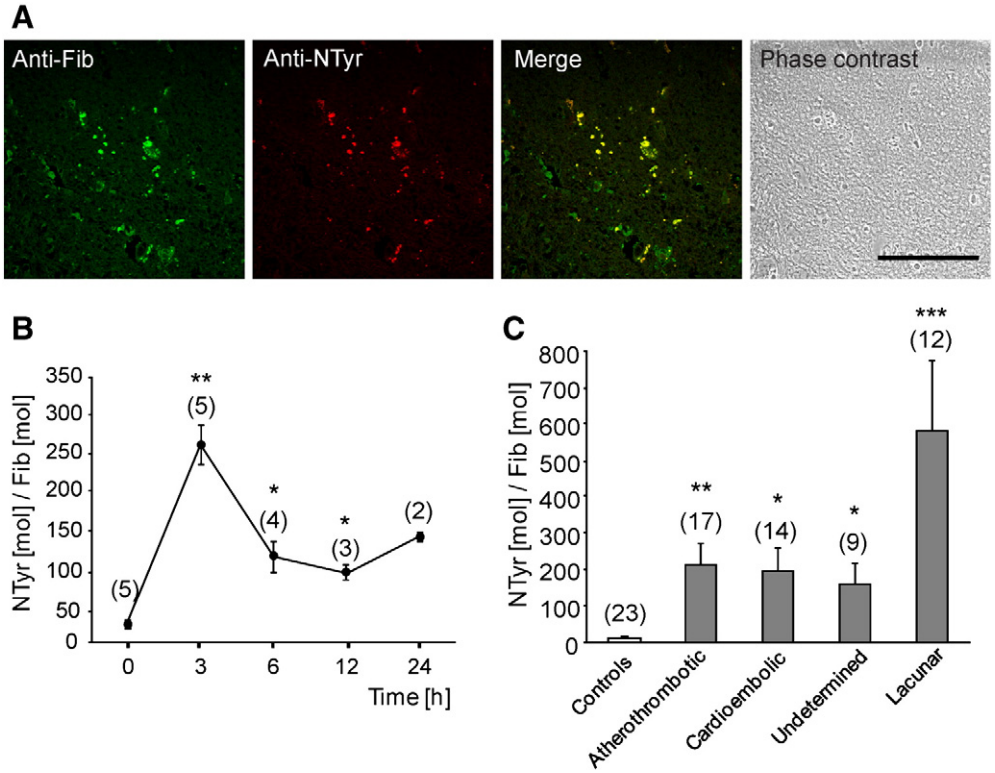

Fig. 1. Fibrinogen is nitrotyrosinated after brain ischemia. (A) Human brain parenchyma from a patient suffering an ischemic stroke was analyzed for nitrotyrosination and fibrinogen with anti-fibrinogen and anti-nitrotyrosination antibodies. Bars are 100 μm. (B) Rats were subjected to MCA occlusion and plasma samples were obtained at 0, 3, 6, 12, and 24 h post-ischemia. Fibrinogen was immunoprecipitated and its nitrotyrosination measured. Data are expressed as the mean ± SEM. The number of animal samples analyzed at each time is indicated in parentheses. Samples were analyzed in duplicate. *$p < 0.05$; **$p < 0.001$ by one-way ANOVA followed by LSD post-hoc analysis. (C) Quantification of plasma fibrinogen nitrotyrosination in controls and atherothrombotic, cardioembolic, undetermined and lacunar stroke patients. Samples were extracted around 3 h after the stroke episode. Data are mean ± SEM. The number of patients is indicated in parentheses. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0005$ by one-way ANOVA followed by LSD post-hoc analysis.

3.2. Nitrotyrosination affects fibrinogen structure and hemostasis

Considering the relevance of fibrinogen in stroke prognosis [14–16] we studied the functional consequences of its nitrotyrosination. Analysis of the shift in intrinsic protein fluorescence, a technique used to detect conformational changes in proteins [19], revealed a reduction in the fluorescence emission of fibrinogen treated with the peroxynitrite donor SIN-1, indicating structural alterations in nitrotyrosinated fibrinogen (Fig. 2A). This finding was confirmed by electron microscopy studies showing that nitrotyrosinated fibrinogen formed globular structures that were clearly different from the needle-shaped geometry observed in non-treated fibrinogen (Fig. 2B).

In order to analyze the impact of fibrinogen nitrotyrosination on blood hemostasis, we incubated human blood samples from healthy individuals with 10 mM SNP, a nitric oxide donor, and 50 μM H_2O_2 as a source of free radicals. This treatment mimics the nitro-oxidative condition that leads to the peroxynitrite burst in the ischemic brain. Both compounds (SNP and H_2O_2) react to form peroxynitrite and the consequent nitrotyrosination was observed in the treated clot samples (Fig. 3C) but not in the control clots (Fig. 3A) or those treated with H_2O_2 alone (Fig. 3E). Nitrative stress also affected the coagulation process (see Fig. S3 for a diagram of the clot formation), as assessed by thromboelastometric analysis (Table 1, Figs. 3B, D and F, Fig. S4). The specific contribution of nitro-fibrinogen to clotting was addressed using the FibTEM test (Table 1), which represents only the fibrin component of the clot because platelets are inhibited. This test is used for the detection of fibrinogen deficiency or fibrin polymerization disorders. The clotting time (CT) measures the fibrin formation time as a result of the proteolytic action of thrombin on fibrinogen (see Fig. S3), which is the initial step of the hemostatic response. The FibTEM analysis showed a significantly higher CT when fibrinogen was nitrotyrosinated ($p < 0.05$), but no differences were observed when H_2O_2 was used alone, indicating that only nitrotyrosination slowed down fibrin formation (Table 1). These results could be due to a global nitrotyrosination of the proteins and factors that act in the coagulation cascade.

The coagulation cascade is composed of the intrinsic and the extrinsic pathways. Both pathways involve a high number of proteins directed to produce the cleavage of prothrombin into the active thrombin (Fig. S3). Therefore we studied the impact of nitro-oxidative stress in the proteins that participate in the coagulation cascade by the study of PTT, which measures the intrinsic pathway, and PT, which measures the extrinsic pathway. Both techniques provide information on the physiological thrombin production. We discarded any significant effect of nitro-oxidative stress in the coagulation cascade since no correlation was found between these two pathways and the levels of nitrotyrosinated fibrinogen in stroke patients (Fig. 4). In this case nitrotyrosinated fibrinogen works just as a parameter of general plasmatic protein nitrotyrosination. The lack of correlation between nitro-oxidative stress and changes in the coagulation cascade was corroborated when plasma samples were nitrotyrosinated *in vitro* (Fig. S5).

Considering that nitrotyrosination might affect various proteins besides fibrinogen (Fig. S3), ExTEM was used to analyze the effect of nitrotyrosination on the clotting process *in toto* (Table 1; Fig. 3). The ExTEM test mildly activates hemostasis *via* the physiological activator tissue factor. The result is influenced by extrinsic coagulation factors, platelets and fibrinogen. A significantly higher CT was obtained when nitrotyrosination was induced with the NO donor SNP plus H_2O_2 (Table 1; $p < 0.01$), confirming the observation that there is a decrease in the rate of fibrin formation under nitrative conditions. Clot formation time (CFT) measures the interaction of the fibrin network with platelets,

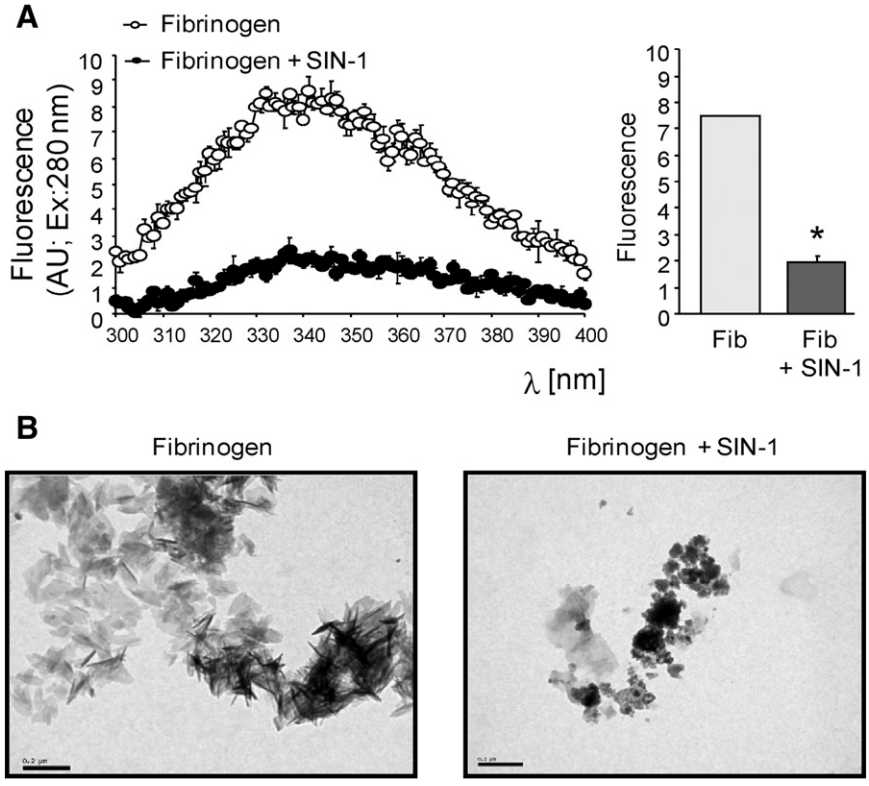

Fig. 2. Nitrotyrosination induces structural changes in fibrinogen. (A) Changes in the intrinsic fluorescent emission signal of nitrotyrosinated fibrinogen. Human fibrinogen (1 mg/mL) was incubated with or without 100 μM SIN-1. Intrinsic fluorescence was measured (Excitation: 280 nm; Emission: 300–400 nm). The maximum differences were recorded at 340 nm and the results are shown as bars representing the mean ± SEM of 4 independent experiments performed in triplicate. *$p < 0.001$ by paired Student's *t*-test. (B) Electron micrographs of fibrinogen incubated with or without 100 μM SIN-1 at 37 °C for 24 h in the dark. Samples were negatively-stained and analyzed by transmission electron microscopy. Bars represent 200 nm.

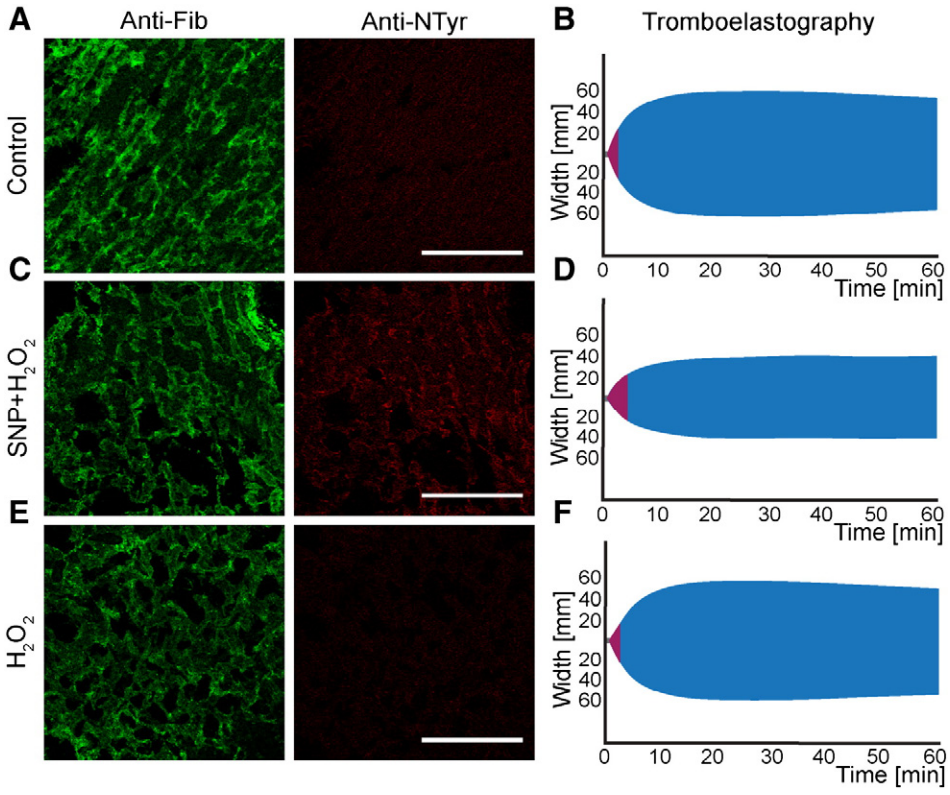

Fig. 3. Nitrotyrosination affects coagulation. Untreated human blood samples (A, B), blood samples treated with 10 mM SNP + 50 μM H_2O_2 (C, D) or treated with 50 μM H_2O_2 alone (E, F) were stained with anti-fibrinogen (left panels) and anti-NTyr (middle panels) antibodies and analyzed by confocal microscopy. B, D and F show the result of the thromboelastography analysis of the clots described in A, C and E. Thromboelastograms show the clotting time (red color) and firmness (blue color) of the clot before plasmin starts to degrade it, measuring between 0 at the initiation of the clotting to 100 mm, assumed as the maximum theoretical firmness. Bars are 100 μm.

Table 1
Thromboelastometric analysis.

	Baseline	SNP + H_2O_2	H_2O_2
FibTEM			
CT [s]	52.7 ± 0.67	63.3 ± 3.4*	48.5 ± 2.5
ExTEM			
CT [s]	57.0 ± 3.1	78.7 ± 3.3**	61.0 ± 3.3
CFT [s]	91.0 ± 5.3	151.3 ± 2.6***	97.5 ± 9.5
MCF [mm]	64 ± 2.3	50.0 ± 4.0*	62.5 ± 4.5
ML [%]	8.3 ± 1.8	1.3 ± 0.9*	9.0 ± 2.0

Coagulation study performed in whole blood from 3 healthy controls. Data correspond to clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF) and maximum lysis (ML) expressed as mean ± SEM. Data were evaluated statistically using one-way ANOVA followed by LSD post-hoc analysis.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

the second step in the hemostatic response. CFT was also significantly higher when nitrotyrosination was induced (Table 1; $p < 0.001$), indicating that protein nitrotyrosination impairs coagulation and also platelet–platelet and platelet–fibrinogen interaction, which fits with the data obtained *in vitro* when platelet aggregation was studied (Fig. S6). Maximum clot firmness (MCF) measures the firmness of the mature clot before plasmin starts to degrade it, the final step in the hemostatic response. A decreased MCF compared to controls was obtained when nitrotyrosination was induced (Table 1; $p < 0.05$). Thromboelastograms also show a decrease in the width of nitrotyrosinated clots (Fig. 3D), an effect directly related to the low firmness of these clots. Analysis of the persistence of the clots over time allowed quantification of the maximum lysis (ML) by measuring fibrinolysis on MCF. The results showed that nitrotyrosination inhibits fibrinolysis (Table 1; $p < 0.05$) making the clots more stable for a longer time. None of the hemostasis steps analyzed were affected by the oxidative conditions induced with H_2O_2 alone. On the other hand, nitro-oxidative stress may affect other factors that also participate in clot lysis.

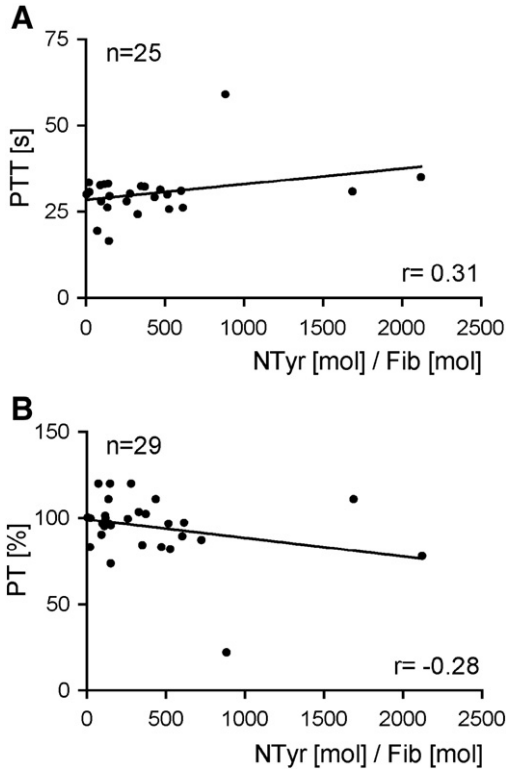

Fig. 4. Study of the effect of ischemic stroke on the coagulation cascade. (A) Partial thromboplastin time (PTT) measures the activity of the intrinsic coagulation cascade. The graph shows the correlation study between PTT and nitro-fibrinogen in stroke patients (n = 25). (B) Prothrombin time (PT) measures the extrinsic coagulation pathway. The graph shows the correlation study between PT and nitro-fibrinogen in stroke patients (n = 29).

3.3. Neuronal effects of fibrinogen nitrotyrosination

Because fibrinogen deposition in the brain of mice seriously damages neurons [27], an assessment was made of the effect of soluble fibrinogen and nitro-fibrinogen on a human neuroblastoma cell line. Following exposure of the cells to both types of fibrinogen, measurements were made of the activation of caspase-3, a key enzyme in the intracellular apoptotic pathway. It was found that at pathophysiological concentrations, both types of fibrinogen activate caspase-3 after 45 min of treatment (Figs. 5A–B), being nitro-fibrinogen a more potent caspase-3 activator than unmodified fibrinogen ($p < 0.05$). These data correlated with viability assays carried out on neuroblastoma cells (Fig. 5C). Nitro-fibrinogen was neurotoxic at all concentrations tested and always more toxic than normal fibrinogen, which was toxic only at 5 and 10 μg/μL.

4. Discussion

In an ischemic stroke the tissue responds with a cascade of events that aims to protect the damaged brain, *e.g.* increased NO production to vasodilate and maintain blood perfusion [28]. Moreover, ischemia results in mitochondrial dysfunction, which in turn leads to a burst in free radical production [8], and the generation of peroxynitrite [29]. Subsequent protein nitrotyrosination, which participates in massive tissue parenchyma damage, will be largely responsible for cell death.

Our findings suggest that the toxic effects of peroxynitrite are not merely confined to the brain tissue affected by the disruption in blood supply, but can target circulating plasma proteins as well. We detected circulating fibrinogen nitrotyrosination as early as 3 h after the first stroke symptoms. Therefore, the profile of fibrinogen nitrotyrosination after brain ischemia seems to mirror the pathophysiological events taking place at the ischemic focus. All subtypes of ischemic stroke (atherothrombotic, cardioembolic, undetermined, and lacunar) show significantly higher values of fibrinogen nitrotyrosination, suggesting that regardless of the causes of the ischemic stroke, all of them share a common mechanism that leads to peroxynitrite production.

Absolute increases in fibrinogen levels have been reported in stroke patients, probably reflecting fibrinogen's up-regulation as an acute-phase reactive protein [30], and has been related to both prognosis and risk factors of stroke [14,13]. Our data suggest that fibrinogen nitrotyrosination induces impairment of the cleavage of nitrotyrosinated fibrinogen by thrombin due to the structural changes of the molecule without detectable changes in thrombin activity *per se*. This impairment also affects the assembly of the fibrinopeptides to form fibrin and the fibrin–fibrin interaction, since the whole tyrosines present in fibrinogen are in the fibrinopeptides. Moreover its binding to platelet is also dramatically affected.

Therefore fibrinogen nitrotyrosination slows down coagulation and makes the clots more resistant to fibrinolysis. This process could be a protective response to modulate coagulation in a way that allows partial perfusion at the early stages of the hemostasis response but stabilizes the clots at longer times. Our observation on the impact of fibrinogen nitrotyrosination on hemostasis may also apply to other ischemic conditions presenting increased nitrotyrosinated fibrinogen such as myocardial infarct due to coronary artery disease [31]. Thus, nitro-fibrinogen can be seen as a rapid unspecific biomarker of acute ischemic processes that may facilitate a rapid and accurate diagnosis of ischemic stroke, reducing the mortality, neurological deficits and systemic damage associated with late diagnosis [32–34].

We found that plasma nitrotyrosination can be prevented with the antioxidant GSH, probably due its scavenging effect, avoiding

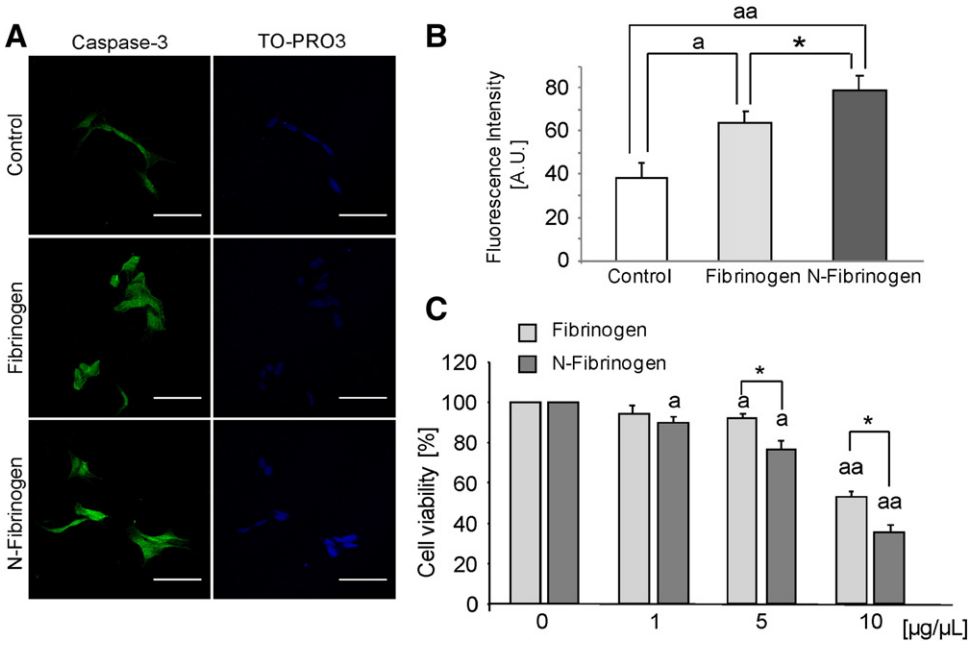

Fig. 5. Fibrinogen and nitro-fibrinogen induce neurotoxicity. (A) Human neuroblastoma cells were treated with 5 μg/μL fibrinogen and nitro-fibrinogen. Caspase-3 activation was studied 45 min after treatment. Bars are 50 μm. (B) Quantification of caspase-3 activation by fibrinogen and nitro-fibrinogen. Data are the mean ± SEM of 4 independent experiments analyzed by one-way ANOVA followed by LSD post-hoc analysis. (a) $p < 0.05$, (aa) $p < 0.005$ in comparison to the controls; *$p < 0.05$ in comparison to fibrinogen data. (C) Human neuroblastoma cells were treated with increasing concentrations of fibrinogen and nitro-fibrinogen. Cell viability was measured by MTT reduction. Data are the mean ± SEM of 6 independent experiments performed in triplicate and analyzed by one-way ANOVA followed by LSD post-hoc analysis. (a) $p < 0.05$, (aa) $p < 0.005$ in comparison to the controls; *$p < 0.05$ in comparison to fibrinogen data.

peroxynitrite formation. The drop in the levels of GSH associated with aging constitutes a major risk factor for stroke [35–37], and polymorphisms in some enzymes related to GSH biosynthesis have been related to a higher risk of stroke [38–40]. Therefore, it is plausible to think that circulating GSH avoids protein nitrotyrosination in basal conditions, and that this protective mechanism might be compromised in subjects that are susceptible of suffering a stroke.

Fibrinogen is also a known pro-inflammatory agent in the brain [41]. The increased permeability of the blood brain barrier during ischemic stroke [42,43] allows extravasation of different plasmatic proteins into the brain parenchyma. Our findings demonstrate the fibrinogen-dependent activation of proapoptotic caspase-3 and neuronal death, an effect enhanced by fibrinogen nitrotyrosination. Together, these experiments suggest that fibrinogen, and especially nitro-fibrinogen, play a relevant role in the progression of brain tissue damage in the penumbra area, determining the magnitude of the final injury.

5. Conclusions

In summary, nitro-fibrinogen may be seen as a Janus molecule that participates in processes that are critical to the management of ischemic stroke. At early stages nitro-fibrinogen has a protective role, delaying clot formation, but in the long term it becomes harmful due to the production of fibrinolysis resistant clots and the induction of neuronal damage. Altogether, the present findings indicate that nitro-fibrinogen is a key molecule in the pathophysiology of ischemic stroke and it would be plausible to consider it as a novel therapeutic target.

Author disclosure statement

The authors declare that they have no competing interests that might be perceived to influence the results and discussion reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.12.007.

References

[1] V.L. Feigin, C.M. Lawes, D.A. Bennett, S.L. Barker-Collo, V. Parag, Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review, Lancet Neurol. 8 (2009) 355–369.
[2] T. Ingall, Stroke: incidence, mortality, morbidity and risk, J. Insur. Med. 36 (2004) 143–152.
[3] J. Kennedy, M.D. Hill, K.J. Ryckborst, et al., Fast assessment of stroke and transient ischaemic attack to prevent early recurrence (FASTER): a randomised controlled pilot trial, Lancet Neurol. 6 (2007) 961–969.
[4] J.C. Chavez, O. Hurko, F.C. Barone, G.Z. Feuerstein, Pharmacologic interventions for stroke: looking beyond the thrombolysis time window into the penumbra with biomarkers, not a stopwatch, Stroke 40 (2009) 558–563.
[5] I. Ferrer, A.M. Planas, Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra, J. Neuropathol. Exp. Neurol. 62 (2003) 329–339.
[6] F.X. Guix, I. Uribesalgo, M. Coma, F.J. Muñoz, The physiology and pathophysiology of nitric oxide in the brain, Prog. Neurobiol. 76 (2005) 126–152.

[7] D.Y. Zhu, Q. Deng, H.H. Yao, et al., Inducible nitric oxide synthase expression in the ischemic core and penumbra after transient focal cerebral ischemia in mice, Life Sci. 71 (2002) 1985–1996.
[8] S. Cuzzocrea, D.P. Riley, A.P. Caputi, D. Salvemini, Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury, Pharmacol. Rev. 53 (2001) 135–159.
[9] K. Niizuma, H. Endo, P.H. Chan, Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival, J. Neurochem. 109 (Suppl. 1) (2009) 133–138.
[10] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 1620–1624.
[11] M. Coma, F.X. Guix, I. Uribesalgo, et al., Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination, Brain 128 (2005) 1613–1621.
[12] F.X. Guix, G. Ill-Raga, R. Bravo, et al., Amyloid-dependent triosephosphate isomerase nitrotyrosination induces glycation and tau fibrillation, Brain 132 (2009) 1335–1345.
[13] A.R. Rudnicka, S. Mt-Isa, T.W. Meade, Associations of plasma fibrinogen and factor VII clotting activity with coronary heart disease and stroke: prospective cohort study from the screening phase of the Thrombosis Prevention Trial, J. Thromb. Haemost. 4 (2006) 2405–2410.
[14] G.J. Del Zoppo, D.E. Levy, W.W. Wasiewski, et al., Hyperfibrinogenemia and functional outcome from acute ischemic stroke, Stroke 40 (2009) 1687–1691.
[15] N.M. Di Napoli, P. Singh, Is plasma fibrinogen useful in evaluating ischemic stroke patients?: why, how, and when, Stroke 40 (2009) 1549–1552.
[16] J.M. Wardlaw, V. Murray, E. Berge, G.J. Del Zoppo, Thrombolysis for acute ischaemic stroke, Cochrane Database Syst. Rev. 4 (2009) (CD000213).
[17] F.J. Pérez-Asensio, X. de la Rosa, F. Jiménez-Altayó, et al., Antioxidant CR-6 protects against reperfusion injury after a transient episode of focal brain ischemia in rats, J. Cereb. Blood Flow Metab. 30 (2010) 638–652.
[18] V. De Filippis, R. Frasson, A. Fontana, 3-Nitrotyrosine as a spectroscopic probe for investigating protein protein interactions, Protein Sci. 15 (2006) 976–986.
[19] J.T. Vivian, P.R. Callis, Mechanisms of tryptophan fluorescence shifts in proteins, Biophys. J. 80 (2001) 2093–2109.
[20] P.I. Moreira, L.M. Sayre, X. Zhu, et al., Detection and localization of markers of oxidative stress by in situ methods: application in the study of Alzheimer disease, Methods Mol. Biol. 610 (2010) 419–434.
[21] R. Pazdro, J.R. Burgess, The antioxidant 3H-1,2-dithiole-3-thione potentiates advanced glycation end-product-induced oxidative stress in SH-SY5Y cells, Exp. Diabetes Res. 2012 (2012) 137607.
[22] J. Xu, L. He, S.H. Ahmed, et al., Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells, Stroke 31 (2000) 1744–1751.
[23] M. Tajes, G. Ill-Raga, E. Palomer, et al., Nitro-oxidative stress after neuronal ischemia induces protein nitrotyrosination and cell death, Oxid. Med. Cell. Longev. 2013 (2013) 826143.
[24] A. Abulrob, E. Brunette, J. Slinn, E. Baumann, D. Stanimirovic, In vivo optical imaging of ischemic blood–brain barrier disruption, Methods Mol. Biol. 763 (2011) 423–429.
[25] G.Y. Yang, A.L. Betz, Reperfusion-induced injury to the blood–brain barrier after middle cerebral artery occlusion in rats, Stroke 25 (1994) 1658–1664.
[26] C. Justicia, A. Martin, S. Rojas, et al., Anti-VCAM-1 antibodies did not protect against ischemic damage either in rats or in mice, J. Cereb. Blood Flow Metab. 26 (2006) 421–432.
[27] J. Paul, S. Strickland, J.P. Melchor, Fibrin deposition accelerates neurovascular damage and neuroinflammation in mouse models of Alzheimer's disease, J. Exp. Med. 204 (2007) 1999–2008.
[28] M.A. Moro, A. Cardenas, O. Hurtado, J.C. Leza, I. Lizasoain, Role of nitric oxide after brain ischaemia, Cell Calcium 36 (2004) 265–275.
[29] R. Taffi, L. Nanetti, L. Mazzanti, et al., Plasma levels of nitric oxide and stroke outcome, J. Neurol. 255 (2008) 94–98.
[30] T. Dziedzic, Clinical significance of acute phase reaction in stroke patients, Front. Biosci. 13 (2008) 2922–2927.
[31] C. Vadseth, J.M. Souza, L. Thomson, et al., Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species, Biol. Chem. 279 (2004) 8820–8826.
[32] L.B. Goldstein, D.L. Simel, Is this patient having a stroke? JAMA 293 (2005) 2391–2402.
[33] D.T. Laskowitz, S.E. Kasner, J. Saver, K.S. Remmel, E.C. Jauch, Clinical usefulness of a biomarker-based diagnostic test for acute stroke: the Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study 49, Stroke 40 (2009) 77–85.
[34] E.H. Yu, C. Lungu, R.M. Kanner, R.B. Libman, The use of diagnostic tests in patients with acute ischemic stroke, J. Stroke Cerebrovasc. Dis. 18 (2009) 178–184.
[35] G.A. Hazelton, C.A. Lang, Glutathione contents of tissues in the aging mouse, Biochem. J. 188 (1980) 25–30.
[36] L. Gil, W. Siems, B. Mazurek, et al., Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes, Free Radic. Res. 40 (2006) 495–505.
[37] P.K. Maurya, S.I. Rizvi, Age-dependent changes in glutathione-s-transferase: correlation with total plasma antioxidant potential and red cell intracellular glutathione, Indian J. Clin. Biochem. 25 (2010) 398–400.
[38] H. Kolsch, M. Linnebank, D. Lutjohann, et al., Polymorphisms in glutathione S-transferase omega-1 and AD, vascular dementia, and stroke, Neurology 63 (2004) 2255–2260.
[39] A. Turkanoglu, D.B. Can, S. Demirkaya, S. Bek, O. Adali, Association analysis of GSTT1, GSTM1 genotype polymorphisms and serum total GST activity with ischemic stroke risk, Neurol. Sci. 31 (2010) 727–734.
[40] B. Voetsch, R.C. Jin, C. Bierl, et al., Promoter polymorphisms in the plasma glutathione peroxidase (GPx-3) gene: a novel risk factor for arterial ischemic stroke among young adults and children, Stroke 38 (2007) 41–49.
[41] K. Akassoglou, S. Strickland, Nervous system pathology: the fibrin perspective, Biol. Chem. 383 (2002) 37–45.
[42] H.S. Han, Y. Qiao, M. Karabiyikoglu, R.G. Giffard, M.A. Yenari, Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation, J. Neurosci. 22 (2002) 3921–3928.
[43] C. Iadecola, F. Zhang, R. Casey, M. Nagayama, M.E. Ross, Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene, J. Neurosci. 17 (1997) 9157–9164.