

Proenkefalin A and protachykinin in ischemic neurological complications after cardiac surgery

Milan Skitek, Aleš Jerin

Institute of Clinical Chemistry and Biochemistry, University Medical Centre Ljubljana, Slovenia
Running title: Skitek et al. Biomarkers in ischemic brain injury

ABSTRACT

Aim The evaluation of biomarkers of acute ischemic brain injury following surgical revascularization of the heart with the use of the heart-lung machine (cardiopulmonary bypass, CPB).

Methods Twenty consecutive patients were divided into two groups: the first 10 patients received a potential neuroprotective human recombinant erythropoietin, while the remaining 10 comprised the control group. Neurological complications were monitored by measuring serum concentrations of neuropeptide proenkephalin A (PENK-A) and protachykinin A (PTA) before and in the first 5 days after surgery, comparing the neurological outcome with MRI examinations.

Results Both the erythropoietin-treated group and control group were comparable with a non-significant difference shown for the postoperative concentrations of PENK-A and PTA. A comparison of serum concentrations of the biomarkers of 16 patients without brain ischemia and 4 patients with acute ischemia also displayed no significant differences, regardless of erythropoietin therapy.

Conclusion In our pilot study the analysis of PENK-A and PTA serum concentrations might not be the strategy to enable the monitoring and evaluation of neuroprotective stroke treatment, but further studies are required to investigate its role in acute ischemic brain injury.

Key words: biomarkers, ischemic brain injury, cardiac surgery

Corresponding author:

Milan Skitek
University Medical Centre Ljubljana,
Institute of Clinical Chemistry and
Biochemistry
Njegoševa ulica 4, SI-1525 Ljubljana,
Slovenia
E-mail: milan.skitek@kclj.si

Original submission:

31 December 2015;

Accepted:

08 January 2016.

doi: 10.17392/845-16

INTRODUCTION

Neurological complications after cardiac surgery and revascularization of the heart with the use of the heart-lung machine (cardiopulmonary bypass, CPB) represent a significant problem in healthcare and stroke is one of the leading causes of death in the world (1,2).

Ischemic stroke is associated with a variety of pathophysiological changes which lead to glial and neuronal brain changes. During the last few years, several biomarkers have been proposed to predict, diagnose and monitor brain injury. Among them the most studied are serum neuron-specific enolase (NSE), protein S-100B and N-methyl-D-aspartate (NMDA) receptor peptides and their antibodies. Biomarkers are associated with infarct size and with damage to the blood-brain barrier (BBB) (3-9).

Recent studies identified stable precursor fragments of the neuropeptides enkephalin (pro-enkephalin A, PENK-A) and substance P (pro-tachykinin A, PTA) as markers of ischemic stroke and BBB integrity and also myocardial infarction (10,11). Both mature neuropeptides have a role as neurotransmitters and evidence suggests a role in immune and nociception stimulation. The distribution of PENK-A includes nervous system, adrenal medulla and immune system. Enkephalins are co-released from nerve terminals with catecholamines. The PENK-A and PTA are involved in some neuropathologies, including Parkinson's disease, Alzheimer's disease, and head injury (12). In contrast to acute cardiac diseases, validated markers for ischemic stroke are lacking. Early detection of patients with increased risk is of great importance, especially in stroke events. The identification of novel markers is of great importance for both diagnostic and prognostic evaluation of patients admitted to the stroke unit, because no current guideline makes recommendations for the use of biomarkers for risk stratification in stroke (13,14). Several recently published reports have described tissue-protective nonhematological effects of recombinant human erythropoietin (rHuEpo) that could have potential protective effects on ischemia-induced tissue damage in several organs. The protective effects of rHuEpo on central and peripheral neurons, cardiomyocytes, hepatocytes, vascular endothelial cells, the pancreas, and uterus have been substantiated. Several

mechanisms of rHuEpo neuroprotection have been recognized, including the decreasing of glutamate toxicity, inducing the generation of neuronal antiapoptotic factors, reducing inflammation, decreasing nitric oxide-mediated injury and direct antioxidant effects (15,16).

The goal of the present study was to determine whether molecular brain biomarkers PENK-A and PTA could identify a lower burden of acute ischemic brain injury following CPB in patients treated with rHuEpo versus control, and whether these molecular biomarkers are consistent with the MRI findings. This study is an unpublished part of the clinical study that has already been published regarding clinical outcome (9,17). We separated the clinical and biochemical parts due to a technical reason (analysis of brain biomarkers PENK-A and PTA was performed in 2 months following the conclusion of the clinical part of the study).

PATIENTS AND METHODS

Patients and study design

We prospectively evaluated 20 patients as a consecutive group undergoing cardiac surgery on CPB. An approval was obtained by the National Medical Ethics Committee of the Republic of Slovenia as well as the written informed consents of all the patients.

Patients under the age of 18, with known malignant hypertension, cancer, hematological disorder, renal or hepatic failure, receiving rHuEpo therapy and allergies to medications were excluded from the study. The patients were assigned to either the erythropoietin treated (Epo) or control group in random mode. The 10 patients in the Epo group received 3 consecutive doses (24,000 IU) of epoetin alfa (Eprex; Janssen-Cilag, Turnhout, Belgium) administered intravenously. The first dose was given 1 day before the procedure, the second dose on the day of operation, and the third dose 1 day after completion of the surgery.

Methods

Patients underwent preoperative testing within 2 days of surgery that included medical history, an electrocardiograph (ECG), an magnetic resonance imaging (MRI) and physical and mental status examination by a neurologist. Blood was collected prior to anesthesia on the day of surgery for

subsequent biomarker analysis. Blood was again collected and MRI examinations performed within 5 days after the surgery (a median of 4 days, range 2 to 5). All patients had a similar intravenously-inhalation anesthesia and operation protocol regarding hypothermia, hemodilution, cardioplegia and monitoring of all relevant clinical parameters (arterial pressure, cardiac arrest, duration of extracorporeal circulation, etc.). A delirium assessment was performed 24 and 48 hours after surgery.

Blood samples were centrifuged within 30 minutes and serum was stored at -20 °C until assayed in duplicate in one batch within 6 months. Concentrations of PENK-A and PTA were determined by ELISA (reagents: Proecephalin A, Cusabio Biotech and Protachykinin-1, Cusabio Biotech; instrument Personal Lab™, Adaltis, Italy). All samples were measured in one batch.

The lower levels of detection were 3.9 ng/L (PENK-A) and 0.078 µg/L (PTA) and the measuring range was set at 15.6 - 1000 ng/L for PENK-A and 0.312 - 20 µg/L for PTA by the manufacturer. The imprecision (coefficient of variation, CV) was according to the specifications of manufacturer.

CV intra-assay: PENK-A < 8% (CV estimated at three samples of known concentration tested twenty times on one plate to assess); PTA < 8% (CV estimated at three samples of known concentration tested twenty times on one plate to assess)

CV inter-assay: PENK-A ≤ 10% (CV estimated at three samples of known concentration tested in twenty assays to assess); PTA ≤ 10% (CV estimated at three samples of known concentration tested in twenty assays to assess).

Magnetic Resonance Imaging (MRI) was performed using standardized protocols on a 3T instrument (Magnetom Trio Tim; Siemens, Erlangen, Germany) (18). Scans were always performed in the same order with a T1-weighted 3-plane localizer, diffusion-weighted imaging (DWI) sequence, and fluid-attenuated inversion recovery (FLAIR) sequence. The images were presented to one of the investigators blinded to the clinical assessments.

Statistical analysis

Nonparametric statistics was used and a comparison using a Mann-Whitney independent test performed. The significant level was set at the level p<0.05.

RESULTS

The baseline characteristics of the erythropoietin-treated (Epo) and control group were comparable in terms of age, sex, blood pressure, diabetes, hyperlipidemia, body mass index, carotid disease, peripheral arterial disease, chronic atrial fibrillation and current smoking. None of the patients had significant carotid lesions. All of the patients underwent complete coronary artery revascularization.

All 20 patients survived open heart surgery. No patient showed neurological dysfunction before the operation. The MRI performed 24 hours before the surgery confirmed chronic multiple small ischemic lesions (2 to 5 mm) in all of the patients. Only 1 patient had a large ischemic region in the left middle artery circulation. It was observed that 4 of 10 patients from the control group had postoperative ischemic brain lesions, two of them larger than 5 mm (cerebrovascular insult). Another two patients in control group showed small postoperative ischemic lesions of about 2 mm in diameter. MRI scans revealed no acute postoperative ischemic brain lesions in patients treated with human recombinant erythropoietin.

The erythropoietin-treated group and control groups were comparable with respect to serum PENK-A and PTA (before and after surgery) according to the comparable study of clinical outcome (Table 1).

Table 1. Comparison of serum preoperative (pre) and postoperative (post) concentrations of biomarkers of the erythropoietin-treated (Epo) group and control group

Parameter (median)	Epo	Control	p*
pre PENK-A (ng/L)	35.10	28.05	
Q1-Q3 (ng/L)	25.35-38.07	21.65-35.55	0.283
post PENK-A (ng/L)	17.70	26.85	
Q1-Q3 (ng/L)	14.125-27.77	15.05-48.70	0.509
pre PTA (µg/L)	0.343	0.383	
Q1-Q3 (µg/L)	0.2048-0.3885	0.3355-0.4275	0.173
post PTA (µg/L)	0.239	0.257	
Q1-Q3 (µg/L)	0.2153-0.3287	0.1835-0.3225	0.934

*10 patients from each group, Mann-Whitney test; PENK-A, proenkephalin A; PTA, protachykinin A; Q1-Q3, interquartile range

Pre- and postoperatively comparison of serum concentrations of biomarkers in 16 patients without brain ischemia and 4 patients with acute postoperative ischemia regardless of erythropoietin therapy also displayed no significant differences (Table 2).

Table 2. Comparison of serum preoperative (pre) and postoperative (post) concentrations of biomarkers of four patients with (ISCH) and 16 patients without brain ischemia (NOISCH) regardless of erythropoietin therapy

Parameter (median)	NOISCH	ISCH	p*
pre PENK-A (ng/L)	34.20	25.45	0.395
Q1–Q3 (ng/L)	26.40–36.70	21.65–34.05	
post PENK-A (ng/L)	24.85	15.05	0.299
Q1–Q3 (ng/L)	15.65–33.65	8.35–38.50	
pre PTA (ng/L)	0.348	0.396	0.342
Q1–Q3 (ng/L)	0.2467–0.3918	0.303–0.4825	
post PTA (ng/L)	0.2310	0.1835	0.342
Q1–Q3 (ng/L)	0.1855–0.3260	0.1635–0.2385	

*Mann-Whitney test; PENK-A, proenkephalin A; PTA, protachykinin A; Q1–Q3, interquartile range

Diagnostic accuracy through ROC analysis proved similar specificity and a predictive value for pre- and post-operative PENK-A and PTA (Table 3).

Table 3. Diagnostic accuracy in optimal cut-off values of proenkephalin A (PENK-A) and protachykinin A (PTA) serum preoperative (pre) and postoperative (post) concentrations of four patients with and 16 patients without brain ischemia

Parameter	Sensitivity (95% CI)	Specificity (95% CI)	+ LR	- LR	+ PV	- PV
pre PENK-A	75.00 (19.4–99.4)	75.00 (47.6–92.7)	3.00	0.33	42.9	92.3
post PENK-A	75.00 (19.4–99.4)	68.75 (41.3–89.0)	2.40	0.36	37.5	91.7
pre PTA	50.00 (6.8–93.2)	86.67 (59.5–98.3)	3.75	0.58	50.0	86.7
post PTA	75.00 (19.4–99.4)	73.33 (44.9–92.2)	2.81	0.34	50.0	91.7

PENK-A: ≤ 26.3 ng/L and ≤ 17.0 ng/L; PTA: ≥ 0.411 μ g/L and ≤ 0.19 μ g/L; CI, confidence interval; LR, likelihood ratio; PV, predictive value;

DISCUSSION

The current protocols for monitoring neurological complications after cardiac operations are not optimal. Besides neurological examination and magnetic resonance imaging (MRI), biomarkers have also attained attention in the last few years (3).

Cerebral complications are very important after cardiac surgery with CPB as the leading cause of disability and morbidity. The etiology of neurological complications from heart surgery is multifactorial, including intraoperative micro- and macroemboli, abnormal cerebral perfusion, reperfusion injury, inflammatory and neurohumoral responses (2). Currently, prediction and diagnosis are based on several clinical, neuroimaging and electrophysiological methods (18). The application of these methods is often difficult because of sedation and the hemodynamic instability commonly seen in critically ill patients (19). Routine application of specific biomarkers

with predictive and diagnostic capabilities could in specific clinical situations represent a low cost alternative that could be more suitable and improve management of cardio- and cerebrovascular patients. The release patterns of neuronal and glial tissue derived proteins before and after acute stroke are associated with the neuroradiological and neurobehavioral consequences of ischemic brain lesions and may be useful in short- and long-term outcome prediction (4). The kinetics of serum protein concentrations correlate and are dependent on the subtype of stroke lesions and do not always reflect the degree of brain damage. On the contrary, they often express complex neuronal-glial interactions as a pathophysiological consequence of ischemic brain lesions (14,19).

The goal of the present study was to investigate the diagnostic value of pre- and postoperative serum levels of PENK-A and PTA for postoperative neurological complications after cardiac surgery by CPB for two groups of patients: an erythropoietin-treated and a control group. Comparison of some of the basic biochemical (pre- and postoperative troponin, lactate) and clinical parameters such as the outcome (number of coronary artery bypass grafts, operative time, cardiac arrest and extracorporeal circulation time, mean blood pressure and transfusion) of both groups also proved the insignificant difference of the two groups (17). Only few clinical studies have studied PENK-A and PTA as markers of stroke (10) and as far as we know this is the first time that such a clinical scenario to monitor neuronal injury was used. The study provides several findings. The erythropoietin-treated group and control group were comparable with respect to serum PENK-A and PTA according to a comparable study clinical outcome such as coronary artery bypass grafts, operative time, transfusion volume and blood pressure values at all stages of operation. The comparison of MRI results, neurological outcome and brain ischemia/injury biomarkers proved an interesting finding especially regarding the untreated control group, where we observed postoperative fresh ischemic lesions in 4 out of the 10 patients without rHuEpo therapy. Two of them had lesions larger than 5 mm and experienced delirium (cerebrovascular insult). Postoperative PENK-A and PTA levels were lower in both groups of participants but the decrease of PENK-A was much larger in the epo

group. Comparison of serum concentrations of biomarkers of 16 patients without brain ischemia and 4 patients with acute ischemia regardless of erythropoietin therapy displayed a nonsignificant difference in pre- and postoperative PENK-A and PTA levels. All concentrations were lower in ischemic patients except for the preoperative PTA, where the concentrations were higher on average.

Diagnostic accuracy in optimal cut-off values of PENK-A serum preoperative (pre) and postoperative (post) concentrations (≤ 26.3 ng/L and ≤ 17.0 ng/L) of 4 patients with and 16 patients without brain ischemia proved relatively low sensitivity and specificity for both (pre- & post-) concentrations of PENK-A (of about 75%). Similar is the case with diagnostic accuracy in optimal cut-off values of PTA serum preoperative (pre) and postoperative (post) concentrations (≥ 0.411 μ g/L and ≤ 0.190 μ g/L), except for the pre PTA specificity of about 87%. The positive predictive values for ischemic stroke is relatively low ($\leq 50\%$) comparing the negative predictive value ($\geq 86.7\%$) for both PENK-A and PTA values.

These findings are in concordance with our previous investigation on the same patient population about the diagnostic value of pre- and postoperative serum levels of N-methyl-D-aspartate (NMDA) receptor antibodies (NR2Ab), S100B protein and neuron-specific enolase (NSE) for postoperative neurological complications after cardiac surgery by CPB (9). The main findings obtained in our new study is that serum PENK-A and PTA are not elevated in ischemic patients compared to studies where PENK-A (but not PTA) are elevated in acute stroke patients against nonischemic events. The elevation of PENK-A correlated with stroke severity and with CT brain lesion size (10). It should be clarified that not all brain injuries from ischemia lead to infarction recognized by MRI. The use of brain biomarkers is helpful because it can show injuries that cannot otherwise be detected by imaging. The results reported here suggest the possibility of a neuroprotective effect of rHuEpo when administered to patients in the perioperative period of cardiac surgery. Despite the 40% difference in stroke incidence between the treated and control groups, the pre- and postoperative serum PENK-A and PTA levels did not significantly differ. PENK-A and PTA are considered markers of blood-brain

barrier (BBB) dysfunction. Ischemic stroke is usually accompanied by BBB dysfunction; however, BBB dysfunction is not necessarily indicative of cerebral ischemia. Leakage of the BBB integrity is one of the main features of stroke pathophysiology. The mature neuropeptides in blood are exposed to rapid degradation in vivo and low stability in vitro, which is not the case with stable PENK-A and PTA (12).

The key limitation of the study was the small study group. The monitoring of biomarkers should also be more frequently performed after cardiac surgery to obtain peak concentrations while the effects on long-term clinical outcome should be followed up. The limitation might be also that this study is an unpublished part of the clinical study that has already been published regarding clinical outcome (9,17). We separated the clinical and biochemical parts due to a technical reason as already mentioned in introduction.

In conclusion, we compared the preoperative, postoperative blood levels of PENK-A and PTA and MRI results of the erythropoietin treated and control group of patients undergoing surgical revascularization of the heart with CPB to diagnose cerebral ischemia and ischemic stroke. Our results regarding diagnostic values of pre- and postoperative serum PENK-A and PTA levels and brain protection with rHuEpo did not prove benefit from medical intervention for the patients known to be at increased risk pre- and postoperatively and did not correlate with MRI.

The question if PENK-A and PTA serum concentrations might be the strategy to enable the monitoring and evaluation of neuroprotective stroke treatment still remains unanswered in our pilot study. Further studies are required to investigate their role in acute ischemic brain injury. Given the significant risk of stroke associated with CPB, future studies involving larger multisite trials are warranted as well as the evaluation of alternative molecular biomarkers (including RNA analysis) to reliably identify ischemic brain injury in this population (20-22).

FUNDING

No specific funding was received for this study.

TRANSPARENCY DECLARATION

Competing interests: None to declare.

REFERENCES

- Selnes OA, McKhann GM. Coronary artery bypass surgery and the brain. *NEJM* 2001; 344:451-2.
- Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TN, Turner MB, Wong ND, Wylie-Rosett J; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics-2011 update: a report from American Heart Association. *Circulation* 2011; 123: e18-209.
- Maas MB, Furie KL. Molecular biomarkers in stroke diagnosis and prognosis. *Biomark Med* 2009; 3:363-83.
- Herrmann M, Ehrenreich H. Brain derived proteins as markers of acute stroke: their relation to pathophysiology outcome prediction and neuroprotective drug monitoring. *Restor Neurol and Neurosci* 2003; 3-4:177-90.
- Anand N, Stead LG. Neuron-specific enolase as a marker for acute ischemic stroke: a systematic review. *Cerebrovasc Dis* 2005; 20:213-19.
- Dassan P, Keir G, Brown MM. Criteria for a clinically informative serum biomarker in acute ischemic stroke: A review of S100B. *Cerebrovasc Dis* 2009; 3:295-302.
- Bokesch PM, Izykenova GA, Justice JB, Easley KA, Dambinova SA. NMDA receptor antibodies predict adverse neurological outcome after cardiac surgery in high-risk patients. *Stroke* 2006; 37:1432-36.
- Weissman JD, Khounteev GA, Heath R, Dambinova SA. NR2 antibodies: risk assessment of transient ischemic attack (TIA)/stroke in patients with history of isolated and multiple cerebrovascular events. *J Neurol Sci* 2011; 300:97-102.
- Skitek M, Jerin A. N-methyl-D-aspartate-receptor antibodies, S100B protein, and neuron-specific enolase before and after cardiac surgery: association with ischemic brain injury and erythropoietin prophylaxis. *Lab Medicine* 2013; 44:56-62.
- Doehner W, Haehling S, Suhr J, Ebner N, Schuster A, Nagel E, Melms A, Wurster T, Stellos K, Gawaz M, Bigalke B. Elevated plasma levels of neuropeptide proenkephalin A predict mortality and functional outcome in ischemic stroke. *J Am Coll Cardiol* 2012; 60:346-54.
- Ng LL, Sandhu JK, Narayan H, Quinn PA, Squire IB, Davies JE, Bergmann A, Maisel A, Jones DJ. Proenkephalin and prognosis after acute myocardial infarction. *Am Coll Cardiol* 2014; 63:280-89.
- Ernst A, Suhr J, Kohrle J, Bergmann A. Detection of stable N-terminal protachykinin A immunoreactivity in human plasma and cerebrospinal fluid. *Peptides* 2008; 29:1201-06.
- Laskowitz DT, Kasner SE, Saver J, Rimmel KS, Jauch EC. Clinical usefulness of a biomarker-based diagnostic test for acute stroke the biomarker rapid assessment in ischemic injury (BRAIN) study. *Stroke* 2009; 40:77-85.
- Whiteley W, Chong W, Sengupta A, Sandercock P. Blood markers for the prognosis of ischemic stroke a systematic review. *Stroke* 2009; 40:e380-89.
- Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* 2004; 35:1732-37.
- Xiong Y, chopp M, Lee CP. Erythropoietin improves brain mitochondrial function in rats after traumatic brain injury. *Neurol Res* 2009; 31:496-502.
- Lakič N, Šurlan K, Jerin A, Meglič B, Curk N, Bunc M. Importance of erythropoietin in brain protection after cardiac surgery: a pilot study. *Heart Surg Forum* 2010; 13:E185-89.
- Barber PA, Hach S, Tippett LJ, Ross L, Merry AF, Milsom P. Cerebral ischemic lesions on diffusion-weighted imaging are associated with neurocognitive decline after cardiac surgery. *Stroke* 2008; 39:1427-1433.
- Saenger AK, Christenson RH. Stroke Biomarkers: Progress and challenges for diagnosis, prognosis, differentiation, and treatment. *Clin Chem* 2010; 56:21-33.
- Rodriguez-Yanez M, Sobrino T, Vazquez-Herrero F, Brea D, Blanco M, Leira R, Castellanos M, Serena J, Vivancos J, Dávalos A, Castillo J. Early biomarkers of clinical-diffusion mismatch in acute ischemic stroke. 2011; 42:2813-U212.
- Cox CR, Sharp FR. RNA-based blood genomics as an investigative tool and prospective biomarker for ischemic stroke. *Neurol Res* 2013; 35:457-64.
- Kuwashiro T, Ago T, Kamouchi M, Matsuo R, Hata J, Kuroda J, Fukuda K, Sugimori H, Fukuhara M, Awano H, Isomura T, Suzuki K, Yasaka M, Okada Y, Kiyohara Y, Kitazono T. Significance of plasma adiponectin for diagnosis, neurological severity and functional outcome in ischemic stroke - Research for Biomarkers in Ischemic Stroke (REBIOS). *Metab-Clin Experim* 2014; 63:1093-103.