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Basic Science

Validation of the ELISA Method for Quantitative Detection of TNF-α in Patients with Intracerebral Hemorrhage

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Abstract

AIM: We aimed to investigate the sensitivity, reproducibility and validity of the commercial ELISA kits for quantitative detection of TNF-α and their potential application for screening purposes in patients with ICH.

METHODS: Analysis of six independent standard series, evaluation of the deviation of the TNF-α concentration in patients with ICH, standard addition and visual analysis of whole UV-Vis spectra were carefully performed.

RESULTS: Low standard deviations of the absorbance were detected for every standard, as well as in the samples of healthy controls and patients with ICH. The standard addition series have also confirmed high sensitivity and reproducibility of the assay, with a congruent shift of the standard curves with the concentration of TNF-α for the added plasma. The visual analyses of the gained spectra have revealed the absence of any matrix effects from the addition of the human plasma in the reconstituted standards.

CONCLUSION: The commercial ELISA kits can be used in the clinical practice for screening purposes of the plasma TNF-α levels in patients with ICH.

Introduction

Tumor necrosis factor-α (TNF-α) is an important pro-inflammatory mediator in both physiological and pathological states, often considered as a central regulator of the inflammatory processes [1]. A vast number of studies have revealed the crucial role of TNF-α in the pathogenesis of inflammation and rheumatic arthritis [2], in the pathogenesis of type-2 diabetes mellitus [3], atherosclerosis, myocardial ischemia and heart failure [4], in the treatment of cancer [5], in neuroinflammation and neurodegeneration [6], as well as in the physiology of ageing [7]. These findings were repeatedly confirmed and commonly cited over the years, and therefore, the necessity for fast and reproducible tests for its detection is important, both from the basic fundamental scientific approach and also from the applicative approach for its future implementation as a potential biomarker.

In the past several years, several studies have shown that TNF-α is one of the key constituents of the inflammatory cascade that follows intracerebral haemorrhage (ICH), immediately after the bleeding [8]. The huge importance of the TNF-α levels in the clinical practice are mainly connected with the contribution of its up-regulation to the increase of the

blood-brain barrier permeability [9] and the formation of the peri-hematomal edema [10], [11], a significant predictor of bad neurological outcome in patients with ICH [12]. TNF-α was also reported as a link between neuroinflammation and excitotoxicity [13], and his role as a predictor of bad neurological outcome in patients was proven in some studies [14].

Bearing in mind the potential role of TNF-α as a predictor for edema formation and bad neurological outcome in patients with ICH, in this study we aimed to investigate the sensitivity, reproducibility and validity of the commercial ELISA kits for quantitative detection of TNF-α and their potential application in screening of the TNF-α levels in patients with ICH.

Methods

All measurements were performed using the Human TNF-alpha Quantikine ELISA Kit, from Quantikine (cat. Number: DTA00C), according to the manufacturer's instructions. To minimize any errors that can arise from using different buffers and incubation times. All measurements were performed on the same microtiter plate, using the same solutions and under careful control of the environmental conditions (especially temperature) by the same person. We have implemented several approaches: analysis of the standard series, evaluation of the deviation of the TNF-α concentration in patients with ICH, standard addition and analysis of whole UV-Vis spectra.

The standard series were made according to the manufacturer's instructions; briefly, the lyophilized TNF-α was reconstructed with ddH_2O to a stock solution of 10,000 pg/mL, and the Calibrator Diluent RD6-35 was used to produce a dilution series with the following concentrations: 500, 250, 125, 61.5, 31.2 and 15.6 pg/mL TNF-α. Six dilution series were prepared to compare the differences in the gained absorbance. Blood plasma TNF-α levels at admission were evaluated in two men patients with ICH (CSS score 3.0 with initial ICH volume of 63 cm^3, and CSS score 7.5 with an initial volume of ICH 70 cm^3, respectively) and two age-matched healthy controls. All participants have given informed consent. For the preparation of the standard addition series, we have diluted the 500 pg/mL standard 1:1 with blood plasma form patients or healthy controls (instead of using the Calibrator Diluent RD6-35), in this way resulting in 250 pg/mL recombinant human TNF-α standard that additionally contains extra TNF-α from the blood plasma. A dilution series was then prepared, resulting in the following concentrations: 250, 125, 61.5, 31.2 and 15.6 pg/mL TNF-α.

The absorbance at 450 nm was recorded using the ELISA microplate reader Spectra Max 190, Molecular Devices. Additionally, in some cases, we have recorded the whole spectra and visualised them with the SoftMax Pro 7 software. All results were statistically analyzed in Microsoft Excel 2016.

Results

Standard series

In this study, we have analysed the absorbance of six standard series. The results concerning the average absorbance values of each standard and their standard deviation are summarized in the next table (Table 1).

Table 1: Average absorbance and standard deviation of the absorbance for each standard in six dilution series

Standards	Concentration (pg/mL)	Number of independent measurements	A_{450} ± SD	Min value of A_{450}	Max value of A_{450}
1	500.0	6	1.1704 ± 0.04	1.1026	1.1878
2	250.0	6	0.7454 ± 0.02	0.7136	0.7699
3	125.0	6	0.4815 ± 0.01	0.4678	0.4906
4	61.5	6	0.3219 ± 0.01	0.3066	0.3451
5	31.2	6	0.1683 ± 0.01	0.1405	0.1904
6	15.6	6	0.0880 ± 0.01	0.0821	0.0983

The curve fit of each of these standard series is represented in Figure 1. As it can be seen, the dependence of the signal on the concentration of TNF-α is not linear, and a trend towards forming a mild plateau can be noticed for the values of TNF-α higher than 300 pg/mL. This trend was detected in each of the standard series. And after our statistical analyses, we have detected that the polynomial regression of second order gives the best curve fit for the dependence between the absorbance and TNF-α concentration. The R^2 values of the polynomial regression model ranged from 0.9921 till 0.9958. Moreover, except the obvious trend for a mild plateau, the increase in the values of the standard deviance can be detected for the higher concentrations of TNF-α. Namely, the range of the absorbance for the standards with low TNF-α concentration was also low (for instance 0.0162 for the standard with a concentration of 15.6 pg/mL) versus the range for the absorbance of the standard with the highest concentration of 500 pg/mL which had eight times bigger range (0.0852). It seems that the sensitivity of the instrumental method is dependent on the concentration of TNF-α. However, it should be noted that except for the standard with 500 pg/mL TNF-α. We have detected a very good congruence of the values of the absorbance for the standards in each of the six independent series, which suggests high reproducibility of the method.

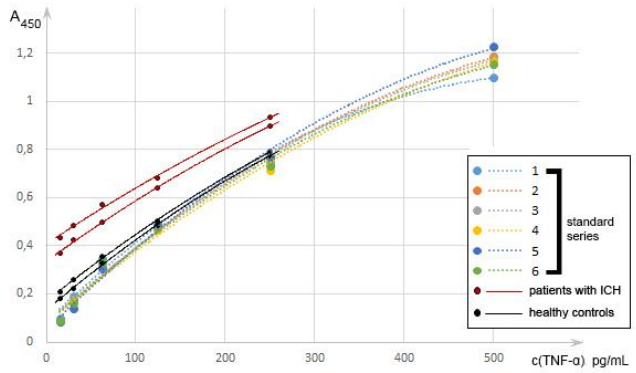

Figure 1: Standard series and series of standard additions using blood plasma of healthy controls and patients with ICH

Deviation of the detected TNF-α concentration in patients and healthy controls

The concentration of TNF-α in patients and healthy controls was estimated in eight independent measurements. The average concentration of TNF-α and the standard deviation of the values for these measurements are presented in Table 2.

Table 2: Deviation of the TNF-α concentration in patients and healthy controls

Subjects	Number of measure-ments	c ± SD	Min value of the concentration	Max value of the concentration
Healthy control 1	8	22.99 ± 0.43 pg/mL	22.36 pg/mL	23.84 pg/mL
Healthy control 2	8	17.41 ± 0.49 pg/mL	16.67 pg/mL	17.87 pg/mL
Patient with ICH 1	8	126.92 ± 0.88 pg/mL	125.82 pg/mL	128.44 pg/mL
Patient with ICH 2	8	158.67 ± 0.68 pg/mL	157.98 pg/mL	159.53 pg/mL

As it can be seen, there is very small deviation of the estimated concentrations of TNF-α both in healthy controls and in patients with ICH. These results indicate high reproducibility of the method.

Standard additions

The series of standard additions using both blood plasma of the healthy controls and patients with ICH have shown a good curve fit (R^2 values of 0.9994 and 0.9995 for the healthy controls; 0.9993 and 0.9963 for the patients with ICH. respectively). The curves possessed the same characteristics as the curves from the standard series: namely, again the polynomial regression of second order was estimated as the best fit for the dependence between the measured signal and the concentration, with the same mild trend for plateau towards the highest TNF-α value. The series with standard addition using plasma from healthy controls have shown a trend for slightly higher values of the absorbance, especially evident for the low standards with 15.6 and 31.2 pg/mL TNF-α, whereas the absorbance of the standard with a high concentration of TNF-α (250 pg/mL) did not show evident differences with the absorbance of the six standard series. In contrast, the series of standard addition using plasma from ICH patients have shown a drastic shift of the curve towards highest values of the absorbance. Having in mind that we have detected quite higher TNF-α level in the blood plasma of the patients, this result only stresses the high sensitivity and reproducibility of the method.

To detect potential matric effects of the added blood plasma to the reconstituted TNF-α standard. we have also recorded and analyzed the whole UV-Vis spectra. Neither of the examined spectra showed any shift of the baseline. All of the spectra were characterized by one sharp peak of the absorbance with λmax of 450 nm. Excluding any matrix effects and proving high sensitivity (Figure 2).

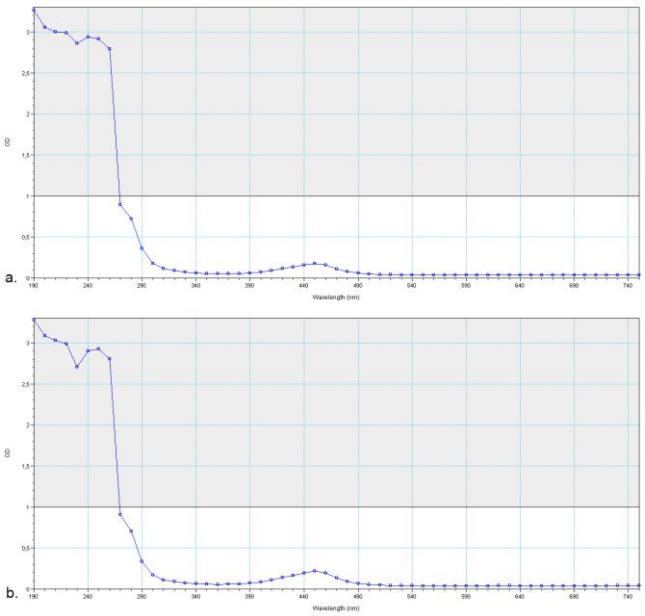

Figure 2: UV-Vis spectra of the standard additions using blood plasma from healthy controls. a) Standard of 15.6 pg/mL (λmax = 0.1787). б) Standard of 31.2 pg/mL (λmax = 0.2205)

Discussion

The detrimental effects of TNF-α towards the secondary brain injury after ICH were well documented in the past years using animal models for ICH [15]. [16]. Recently, Behrouz (2016) [8] have proposed a model explaining that the produced thrombin levels after ICH trigger up-regulation and release of TNF-α in the brain tissue and thus. contribute to the secondary brain injury.

Several studies have also evaluated the peripheral TNF-α level in patients with ICH. For instance, increased peripheral levels of TNF-α were detected in patients with bad neurological outcome. when compared with the patients with a good

outcome [17]. Yang and Shao (2016) [18] have detected higher TNF-α levels in patients with ICH when compared to healthy controls with a peak in the concentration three days after ICH. In both of these studies, commercial ELISA kits were used for the quantitative detection of TNF-α.

In the present study, we have applied several methods to examine the specificity and the reproducibility of the ELISA method for quantitative detection of TNF-α. The literature data for the studies of this type are rare, and especially in the case of patients with ICH or ischemic stroke. For instance, hitherto low-cost sandwich ELISA procedures were validated for the measurement of TNF-α in bovine plasma [19] and the early stages of heart failure [20]. However, the analyses of the whole spectra and evaluation of the matrix effects have not been performed in any of these studies.

In this study, we have shown high reproducibility of the ELISA method for detection of TNF-α. Low standard deviations of the absorbance were detected for every standard in the series, as well as in the samples of healthy controls and patients with ICH. The standard addition series have also confirmed high sensitivity and reproducibility, with a reasonable shift of the standard curves according to the concentration of TNF-α for the added plasma.

The visual analyses of the gained spectra have revealed the absence of any matrix effects from the addition of the human plasma in the reconstituted standards. All of these results suggest that the commercial ELISA kits can be used in the clinical practice for the screening of the TNF-α level in patients with ICH.

We believe that the implementation of this assay could be beneficial for the prediction of the volume of the oedema and the neurological outcome of the ICH patients, which will we in the focus of our next studies?

References

1. Esposito E and Cuzzocrea S. TNF-Alpha as a Therapeutic Target in Inflammatory Diseases. Ischemia- Reperfusion Injury and Trauma. Curr Med Chem. 2009; 16(24): 3152–67. https://doi.org/10.2174/092986709788803024 PMid:19689289

2. Matsuno H. Yudoh K. Katayama R. Nakazawa F. Uzuki M. Sawai T. Yonezawa T. Saeki Y. Panayi GS. Pitzalis C. Kimura T. The role of TNF-alpha in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera. Rheumatology (Oxford). 2002;41(3):329-37. https://doi.org/10.1093/rheumatology/41.3.329

3. Swaroop JJ. Rajarajeswari D. Naidu JN. Association of TNF-α with insulin resistance in type 2 diabetes mellitus. The Indian Journal of Medical Research. 2012;135(1):127-130. https://doi.org/10.4103/0971-5916.93435 PMid:22382194 PMCid:PMC3307173

4. Kleinbongard P. Heusch G. Schulz R. TNFalpha in atherosclerosis. myocardial schemia/ reperfusion and heart failure. Pharmacol Ther. 2010;127(3):295-314. https://doi.org/10.1016/j.pharmthera.2010.05.002 PMid:20621692

5. van Horssen R. Ten Hagen TL. Eggermont AM. TNF-alpha in cancer treatment: molecular insights. antitumor effects. and clinical utility. Oncologist. 2006;11(4):397-408. https://doi.org/10.1634/theoncologist.11-4-397 PMid:16614236

6. Frankola KA. Greig NH. Luo W. Tweedie D. Targeting TNF-α to elucidate and ameliorate neuroinflammation in neurodegenerative diseases. CNS Neurol Disord Drug Targets. 2011;10(3):391-403. https://doi.org/10.2174/187152711794653751 PMid:21288189 PMCid:PMC4663975

7. Bruunsgaard H. Skinhøj P. Pedersen AN. Schroll M. Pedersen BK. Ageing. tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. Clin Exp Immunol. 2000;121(2):255-60. https://doi.org/10.1046/j.1365-2249.2000.01281.x PMid:10931139 PMCid:PMC1905691

8. Behrouz R. Re-exploring Tumor Necrosis Factor Alpha as a Target for Therapy in Intracerebral Hemorrhage. Transl Stroke Res. 2016;7(2):93-6. https://doi.org/10.1007/s12975-016-0446-x PMid:26762364

9. McCoy MK. Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. Journal of Neuroinflammation. 2008;5:45. https://doi.org/10.1186/1742-2094-5-45 PMid:18925972 PMCid:PMC2577641

10. Vakili A. Mojarrad S. Akhavan MM. Rashidy-Pour A. Pentoxifylline attenuates TNF-α protein levels and brain edema following temporary focal cerebral ischemia in rats. Brain Res. 2011;1377:119–25. https://doi.org/10.1016/j.brainres.2011.01.001 PMid:21219888

11. Huang LQ. Zhu GF. Deng YY. Jiang WQ. Fang M. Chen CB. et al. Hypertonic saline alleviates cerebral edema by inhibiting microgliaderived TNF-α and IL-1β-induced Na-K-Cl cotransporter up-regulation. J Neuroinflammation. 2014;11:102. https://doi.org/10.1186/1742-2094-11-102 PMid:24916922 PMCid:PMC4080704

12. Gebel JM Jr. Jauch EC. Brott TG. Khoury J. Sauerbeck L. Salisbury S. Spilker J. Tomsick TA. Duldner J. Broderick JP. Relative edema volume is a predictor of outcome in patients with hyperacute spontaneous intracerebral hemorrhage. Stroke. 2002;33(11):2636-41. https://doi.org/10.1161/01.STR.0000035283.34109.EA

13. Olmos G. Llado J. Tumor necrosis factor Alpha: A link between Neuroinflammation and Excitotoxicity. Mediators of Inflammation. 2014; 2014: 861231. https://doi.org/10.1155/2014/861231 PMid:24966471 PMCid:PMC4055424

14. Silva Y. Leira R. Tejada J. Lainez JM. Castillo J. Davalos A: Molecular signatures of vascular injury are associated with early growth of intracerebral hemorrhage. Stroke. 2005;36:86-91. https://doi.org/10.1161/01.STR.0000149615.51204.0b PMid:15550687

15. Zhang XM. Li XL. Tang SH. Liu QC. Effect of head hypothermia on serum inflammatory cytokines levels in patients with hypertensive intracerebral hemorrhage. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue. 2006; 18(5):294–296. PMid:16700996

16. Hua Y. Wu J. Keep RF. Nakamura T. Hoff JT. Xi G. Tumor necrosis factor-alpha increases in the brain after intracerebral hemorrhage and thrombin stimulation. Neurosurgery. 2006;58:542–5. https://doi.org/10.1227/01.NEU.0000197333.55473.AD PMid:16528196

17. Castillo J. Dávalos A. Alvarez-Sabín J. Pumar JM. Leira R. Silva Y. Montaner J. Kase CS. Molecular signatures of brain injury after intracerebral hemorrhage. Neurology. 2002 26;58(4):624-9.

18. Yang G. Shao GF. Elevated serum IL-11. TNF α. and VEGF expressions contribute to the pathophysiology of hypertensive intracerebral hemorrhage (HICH). Neurol Sci. 2016;37(8):1253-9. https://doi.org/10.1007/s10072-016-2576-z PMid:27115896

19. Farney JK. Mamedova LK. Godsey BH. Bradford BJ. Technical note: validation of an ELISA for measurement of tumor necrosis factor alpha in bovine plasma. J Dairy Sci. 2011;94(7):3504-9. https://doi.org/10.3168/jds.2010-4082 PMid:21700038

20. Creve S. Validation of the analytical methods for the detection of cytokines in the early stages of heart failure. Master thesis. Ghent University. Faculty of Pharmaceutical Sciences. Ghent. 2010.