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

Vladimir Rendevski1*, Boris Aleksovski2, Dragan Stojanov3, Ana Mihajlovska-Rendevska4, Vasko Aleksovski5, Natalija Baneva–Dolnenec6, Dijana Nikodijevic5, Dance Gudeva-Nikovska6

1University Clinic for Neurosurgery, Medical Faculty, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; 2Faculty of Natural Sciences and Mathematics, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; 3Center of Radiology, Clinical Center Niš, Faculty of Medicine, University of Niš, Niš, Serbia; 4University Clinic for Radiology, Medical Faculty, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; 5University Clinic for Neurology, Medical Faculty, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; 6Ministry of Health, Global Fund, Skopje, Republic of Macedonia

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 reconstituted with ddH₂O 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 cm³ and CSS score 7.5 with an initial volume of ICH 70 cm³, 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                                  | 1.704 ± 0.04 | 1.1026           | 1.1878           |
| 2         | 250.0                 | 6                                  | 0.7454 ± 0.02 | 0.7136           | 0.7699           |
| 3         | 125.0                 | 6                                  | 0.4615 ± 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                                  | 0.0990 ± 0.01 | 0.0921           | 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 R² 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 deviation 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.
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 matrix 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).

**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.

| Subjects                  | Number of measurements | 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 | 157.99 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 (R² 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

**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 edema and the neurological outcome of the ICH patients, which will we in the focus of our next studies?

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