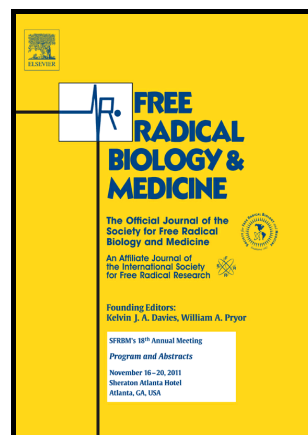


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A high-sensitivity electrochemiluminescence-based ELISA for the measurement of the oxidative stress biomarker, 3-nitrotyrosine, in human blood serum and cells

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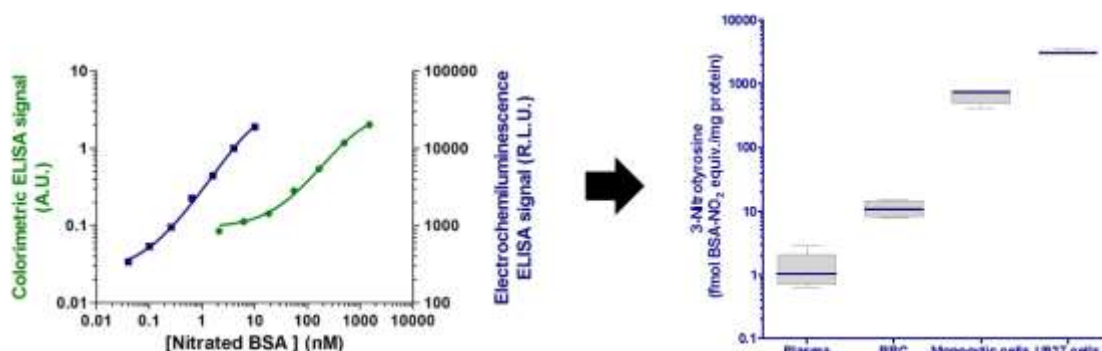
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Abstract

The generation of 3-nitrotyrosine, within proteins, is a post-translational modification resulting from oxidative or nitrative stress. It has been suggested that this modification could be used as a biomarker for inflammatory diseases. Despite the superiority of mass spectrometry-based determinations of nitrotyrosine, in a high-throughput clinical setting the measurement of nitrotyrosine by an enzyme-linked immunosorbent assay (ELISA) is likely to be more cost-effective. ELISAs offer an alternative means to detect nitrotyrosine, but many commercially available ELISAs are insufficiently sensitive to detect nitrotyrosine in healthy human serum. Here, we report the development, validation and clinical application of a novel electrochemiluminescence-based ELISA for nitrotyrosine which provides superior sensitivity (e.g. a 50-fold increase in sensitivity compared with one of the tested commercial colorimetric ELISAs). This nitrotyrosine ELISA has the following characteristics: a lower limit of quantitation of 0.04 nM nitrated albumin equivalents; intra- and inter-assay coefficients of variation of 6.5% and 11.3%, respectively; a mean recovery of $106 \pm 3\%$ and a mean linearity of 0.998 ± 0.001 . Far higher nitration levels were measured in normal human blood cell populations when compared to plasma. Mass spectrometry was used to validate the new ELISA method. The analysis of the same set of chemically modified albumin samples using the ELISA method and mass spectrometry showed good agreement for the relative levels of nitration present in each sample. The assay was applied to serum samples from patients undergoing elective surgery which induces the human inflammatory response. Matched samples were collected before and one day after surgery. An increase in nitration was detected following surgery (median (IQR): 0.59 (0.00-1.34) and 0.97 (0.00-

1.70) nitrotyrosine (fmol of nitrated albumin equivalents/mg protein) for pre- and post-surgery respectively. The reported assay is suitable for nitrotyrosine determination in patient serum samples, and may also be applicable as a means to determine oxidative stress in primary and cultured cell populations.

Graphical abstract



Keywords: 3-nitrotyrosine, oxidative stress, nitrative stress, peroxynitrite, inflammation, human serum, enzyme-linked immunosorbent assay, mass spectrometry

Highlights:

- A highly sensitive ELISA for measurement of 3-nitrotyrosine has been developed
- The new ELISA was validated by mass spectrometry
- Nitration levels in human blood cell populations were higher than in plasma
- An increase in serum nitration after surgery was observed in patients

Abbreviations: Electrochemiluminescence (ECL), Meso Scale Discovery (MSD), nitrated BSA (BSA-NO₂) and lower limit of quantification (LLOQ).

Introduction

Tyrosine nitration is the replacement of the C₃ hydrogen atom, on the aromatic ring of tyrosine, with a nitro group [1], forming 3-nitrotyrosine. This modification is considered to result from oxidative stress and occurs with both free tyrosine (to generate free nitrotyrosine) and tyrosine within a polypeptide sequence (to produce the protein residue adduct, nitrotyrosine). There are several pathways leading to nitration in biological systems, but reactive oxygen species (ROS) are invariably involved and many of the described pathways involve a two-step process [2] containing, first, the formation of a tyrosine radical and, secondly, the reaction of the tyrosine radical with the free radical nitrogen dioxide ($\cdot\text{NO}_2$).

The formation of nitrotyrosine in human tissues/bio-fluids is associated with a number of human diseases which involve a strong inflammatory component. In inflammatory environments, two key nitrating entities, peroxynitrite [3, 4] and the myeloperoxidase-H₂O₂-NO₂⁻ system [5, 6], are both induced. This suggests that nitrotyrosine could be measured as a marker of oxidative stress in clinical studies. For example, plasma protein-associated nitrotyrosine concentrations are increased in patients with systemic lupus erythematosus [7], celiac disease [8], rheumatoid arthritis [9] and cardiovascular disease [10, 11], when compared to healthy control individuals. Increased levels of nitrotyrosine have also been observed in aortic ring tissue from rats following

pharmacological treatment [12] and in mouse plasma after exposure to stressful environmental conditions, such as traffic noise [13].

Some studies have shown that plasma nitrotyrosine levels correlate with disease activity [14-16] and decrease following drug therapy (e.g. statin and rosuvastatin for cardiovascular conditions) [10, 17]. However, the current evidence that oxidative protein modifications are useful biomarkers of disease is compromised by the use of poorly validated methods and the limited number of large clinical studies. The nitrotyrosine modification is no exception to these problems [18]. When measuring nitrotyrosine there are two major methodological issues to consider: (1) low concentrations of the analyte and (2) artefactual formation of nitrotyrosine during sample processing [19]. There have been two main approaches to quantifying nitrotyrosine levels in human plasma and other bio-fluids: ELISAs and GC/LC-MS/MS. These methods have both advantages and disadvantages. The existing ELISA methods for nitrotyrosine are high throughput, relatively cheap and do not involve extensive sample preparation, but they have low sensitivity and are often semi-quantitative. Issues of specificity must also be considered as antibodies might cross-react with other oxidative modifications present, such as chlorotyrosine or nitrophenylalanine [20, 21]. GC/LC-MS/MS is quantitative, highly sensitive and accurate but is low-throughput, time consuming and expensive, limiting this method's usefulness in a clinical setting. The risk of nitrotyrosine formation during sample derivatization for gas chromatography is also a potential confounding factor [22, 23].

For the measurement of nitrotyrosine in a large number of clinical samples, the high-throughput capacity of an ELISA is preferable. Nonetheless, limitations with existing

nitrotyrosine ELISAs need to be addressed before an ELISA can be recommended as a prognostic tool in a clinical setting. Although earlier studies have reported the development of ELISAs for nitrotyrosine [8, 24, 25] and commercial nitrotyrosine assays are widely available and have limits of detection in the range of 1-2 nM, an obstacle still to be addressed is poor sensitivity of these assays. Indeed, existing ELISA methods are often unable to detect nitrotyrosine in a high proportion of human serum samples.

Therefore a high-throughput assay based on a highly sensitive electrochemiluminescence (ECL) platform was developed for nitrotyrosine measurement. The ECL method uses an electrical current to produce light. The base of the ELISA plate contains carbon electrodes which transmit the electrical current to the antibody/antigen complex. The streptavidin tag is conjugated to a ruthenium complex which undergoes redox cycling, following electrical stimulation, resulting in the production of light. Due to the low background and amplification of light signals, through excitation cycles, this platform is highly sensitive [26].

Here we report a novel ELISA for nitrotyrosine which has been analytically validated by LC-MS/MS. We used the assay to compare nitrotyrosine concentrations in normal human plasma, isolated blood cells and cultured cells. We have also applied the assay to serum samples from patients undergoing major elective surgery (as an exemplar of the acute inflammatory response in humans).

Materials and Methods

Reagents

Ultra-pure water was used for buffer preparations throughout. The following reagents were from Sigma Aldrich (Gillingham, UK): phosphate buffered saline powder (PBS; product number P5368), bovine serum albumin (BSA; fraction V, $\geq 96\%$ pure), acetonitrile (anhydrous, 99.8%), iodoacetamide (BioUltra), trypsin (proteomics grade), radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail (product number P8340: pre-made solution of 4-(2-aminomethyl) benzenesulfonyl fluoride hydrochloride at 104 mM, aprotinin at 80 μM , bestatin at 4 mM, E-64 at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM) and Percoll (lot 10226958).

Nitrated bovine serum albumin (nitrated BSA, prepared using tetranitromethane as a nitrating reagent), was purchased from Enzo Life Sciences (New York, USA). Dextran, batch DB4311, was from Pharmacosmos, Holbæk, Denmark. Methanol (analytical reagent grade), acetic acid (1M solution), ammonium bicarbonate (analytical reagent grade), dithiothreitol (product number P2325, 1 M aqueous solution), formic acid (LC-MS grade), Tween-20, foetal bovine serum (FBS, lot 41F6547K) and HyClone™ antibiotic-antimycotic solution (lot J140011) were supplied by Fisher Scientific, Loughborough, UK. Pierce protein-free blocking buffer, in PBS, was purchased from Thermo Scientific (Cramlington, UK). Dulbecco's modified Eagle's medium (DMEM), high glucose DMEM, and L-glutamine were supplied by Lonza (Wolverhampton, UK). Coomassie brilliant blue was purchased from LKB Produkter (Stockholm, Sweden). Acetonitrile (HiPerSolv Chromanorm UPLC grade) was from VWR (West Sussex, UK).

The primary antibody (monoclonal mouse anti-nitrotyrosine, clone number CC.22.8C7.3) and secondary antibody (biotinylated monoclonal mouse anti-nitrotyrosine, clone number CC.22.8C7.3) were from Cambridge Biosciences (Cambridge, UK). “Blocker A” (product number R93BA-4), SULFO-TAG labelled streptavidin and “Read Buffer T 4x” were purchased from Meso Scale Discovery (MSD), Maryland, USA.

Human peripheral blood samples

The nitrotyrosine assay was applied to healthy human plasma and isolated human blood cells. The volunteer blood donors gave informed consent, and this study was approved by the Institutional Research Ethics Committee - approval number 2014/781. For the separation of cells from normal human peripheral blood, the following protocol was used as previously described [27]. The whole blood (approx. 5 ml) was collected into EDTA tubes and centrifuged for 20 min at 350 g, and the plasma was removed. Red blood cell sedimentation was achieved by incubating the cells with dextran (6% w/v), for 30 min at room temperature. The peripheral blood mononuclear cells were isolated using a discontinuous Percoll gradient [27]. Centrifugation through the gradient (20 min, 720 g, 4°C) resulted in mononuclear cells being retained at the upper boundary (between the 68 and 55% Percoll).

In order to study the effect of the surgery-induced inflammatory response on nitrotyrosine levels, serum was collected from 35 patients (18 male, 17 female; 59 ± 13 years old) prior to major elective surgery and again 24 hours after surgery. Clotted blood samples were collected from each patient, at each of the two time points, and

stored frozen in a study approved by the National Research Ethics Service Committee South Central (Research Ethics Committees ref 06/Q1702/152). Prior informed consent to take part in the study was obtained from each individual. The types of major elective surgery undertaken were as follows: resection (thyroid, bowel, colon, stomach, gall bladder or liver – 10 patients), hernia repair (5 patients), kidney, gall or bladder stone removal (5 patients), unknown (5 patients), removal of bladder/urinary diversion (4 patients), treatment of fistulas (3 patients), tumour removal (1 patient), laparoscopic examination of abdominal cancer (1 patient) and treatment of severe claudication (1 patient). C-reactive protein (CRP) was measured at the Clinical Chemistry department, Royal Devon and Exeter (RD&E) Hospital, using an immunoturbidimetric assay based on the analytical platform of a Roche Cobas automated analyser (Roche Diagnostics, West Sussex UK). The CRP assay had a range of 0.3-350 mg/L. Clinical information including white blood cell count (counts for each cell type), blood creatinine and blood urea, was available for some of the patients (n=26 pre-surgery and n=16 post-surgery; 14 matched pairs).

Cell culture

Human U937 cells (a histiocytic lymphoma cell line) were obtained from the ECACC (European Collection of Authenticated Cell Cultures) via Sigma-Aldrich (Gillingham, UK, lot 11D008). U937 cells [28] were cultured in DMEM supplemented with 10% foetal bovine serum, 4 mM L-glutamine and an antibiotic/antimycotic solution (100 units/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B). These non-adherent cells were pelleted by centrifugation at 450 g for 5 min. The media was then removed and the cells were washed twice with PBS (450 g for 5 min).

New electrochemiluminescence-based ELISA method

Preparation of standards: nitrated BSA was aliquoted into 1 μM stock solutions (reconstituted with distilled water) prior to use. The PBS solution contained 10 mM phosphate, 138 mM NaCl and 2.7 mM KCl (pH 7.4). A 1 μM stock was then diluted with dilution buffer (1% Blocker A in PBS) to a concentration of 10 nM to form the highest data point of the standard curve (standard range 0.04 nM–10 nM).

Preparation of plasma, serum and isolated cell samples: Immediately prior to use, plasma/serum samples were defrosted and kept on ice. The plasma/serum was diluted 1 in 5 with dilution buffer (1% Blocker A in PBS) immediately prior to use. All cells were lysed in order to measure cellular protein nitration. Human red blood cells were lysed by hypotonic lysis. Human peripheral blood mononuclear cells and U937 cells were lysed using RIPA buffer with a protease inhibitor cocktail, according to the manufacturer's instructions.

Electrochemiluminescence-based sandwich ELISA protocol: "Standard bind" single spot 96-well plates were purchased from MSD (Maryland, USA) and the platform for analysis of the plates was an ECL Sector Imager 2400 (MSD, Maryland, USA). Primary antibody (non-biotinylated, diluted in PBS to 1 $\mu\text{g}/\text{ml}$) was pipetted into each well of the plate and then incubated at 4°C overnight. The plate was then blocked with 150 μl blocking buffer (3% Blocker A in PBS), at 25°C for one hour with gentle agitation (Jeio Tech Lab Companion SI-300R shaking incubator, Meadowrose Scientific Limited West Sussex, UK). The plate was then washed three times with wash buffer (0.05% Tween-20 in PBS). Each standard/sample (25 μl) was added to the relevant well, with

quadruplicate repeats of each, and incubated for another hour. The plate was washed as above. Each well was incubated with the biotinylated secondary antibody (2 µg/ml in dilution buffer) followed by another wash step and incubation with the streptavidin tag for an hour (1:500 in dilution buffer; streptavidin-coupled to a ruthenium complex). After a final wash, the Read Buffer (150 µl, 2x concentration obtained by dilution with dH₂O) was placed into each well and the plate immediately read on the MSD Sector Imager 2400. The standard curve was plotted and the sample concentrations calculated from these known values. The results were expressed using the unit “nitrated BSA equivalents”, which represents the protein concentration of the commercial nitrated BSA sample which produced an ECL signal equivalent to that of the unknown sample.

Validation of the new ECL-based ELISA

Lower limit of quantification and coefficient of variation: The lower limit of quantification (LLOQ) was defined using the US Food and Drug Administration definition of: the lowest standard with a mean accuracy of 80-120% and a duplicate variation of <20% [29]. The coefficient of variation (CV) was determined by preparing and measuring the same sample multiple times within and across plates (n=8). $CV\% = (\text{standard deviation}/\text{mean}) \times 100\%$. The inter-assay CV for room temperature incubations compared to incubations at a constant temperature of 25°C was also assessed.

Recovery and linearity: A plasma sample was spiked with 1 nM of commercially nitrated BSA (nitrated BSA; Enzo Life Sciences) and diluted to four different concentrations (1 in 2, 1 in 5, 1 in 10 and 1 in 20) to determine the linearity and recovery of the assay.

Antibody specificity: Control experiments were performed, involving both pre-incubation (up to 1 hour) of the detection antibody with 120 μM free 3-nitrotyrosine, and spiking of a nitrated BSA sample with 120 μM free 3-nitrotyrosine. The antibody has been previously characterised in relation to affinity and cross-reactivity [21, 30]. The antibody was reported to have the following cross-reactivities with amino acids similar to 3-nitrotyrosine: 4-nitrophenylalanine: 0.1%; L-tyrosine: 6.2%; 3,5-dinitrotyrosine 1.2% [21]. The manufacturer (Cayman Chemical) states a cross-reactivity with chlorotyrosine of <5%.

Validation of the ECL-based ELISA by mass spectrometry: In order to prepare chemically modified BSA samples, BSA (15 μM) in a bicarbonate buffer (25 mM NaHCO_3 and 100 mM KH_2PO_4) was exposed to 3, 1.5, 0.75 and 0.3 mM peroxyntirite (synthesized according to the method described in reference [31]) to cause nitration of tyrosine residues. A 1 mL sample of the BSA, which had been exposed to 3 mM peroxyntirite, was diluted with 800 μL PBS. This sample was then mixed with sodium dithionite (200 μL of 1.4 M stock solution; final concentration 140 mM), added as 10 μL drops every 2 min, whilst being stirred on ice (final BSA concentration 7.5 μM). The solution was incubated on ice (with stirring) for a further 30 min, to chemically reduce the nitro groups to amino groups [25]. The solution was then transferred into an Eppendorf tube and centrifuged for 5 min (14,000 g , 2 $^\circ\text{C}$). The supernatant was collected into a pre-cooled tube and stored at -20°C until use.

In preparation for mass spectrometry, native (unmodified), nitrated (by peroxyntirite) and chemically reduced BSA samples were run on an SDS-PAGE, and the bands excised and digested with trypsin as previously described [32]. Peptides were separated on an

Ultimate 3000 high-pressure liquid chromatography system (Dionex, UK) coupled to a 5600 Triple TOF mass spectrometer (Sciex, Warrington UK). The peptides were separated on a nano-HPLC column (0.075 x 150 mm 3 μ m PepMap C18, Thermo Scientific, Hemel Hempstead, UK) at a flow rate of 300 nL/min using a gradient elution running from 2% - 45% aqueous acetonitrile (0.1% formic acid) over 60 min. Ionisation of the peptides was achieved with the following settings: spray voltage 2.4 KV, source temperature 150°C, declustering potential 50 V and a curtain gas 15. High resolution TOF MS mode was used to collect survey scans in positive mode from 400 to 1200 Da for 200 ms. MS/MS data was collected using information-dependent acquisition with the following criteria: the 10 most intense ions with +2 to +5 charge states and a minimum intensity of 500 counts-per-second was chosen for analysis, using dynamic exclusion for 20 s, 250 ms acquisition time, and standard settings for rolling collision energy.

The generated data were analyzed using Mascot Daemon statistical software v 2.3.2 (Matrix Science, London, UK [33]) with the following search parameters: type of search: MS/MS Ion Search; enzyme: trypsin; variable modifications: carbamidomethyl (C), oxidation (M), nitro (Y) and nitro (W); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance \pm 0.1 Da; fragment mass tolerance: \pm 0.1 Da; max missed cleavages: 2; instrument type: ESI QUAD TOF. Additional searches were performed with amino (Y) or oxidation (C) as variable modifications, in place of nitro (W). Matches for bovine albumin were then examined for the listed modifications.

The relative amounts of the nitrated peptides were calculated using the Peak View software (AB SCIEX Version 2.0 [34]). Peptide extracted ion chromatograms (XIC), were generated at the m/z of the peptides containing either one/two unmodified

tyrosines, or one/two nitrated tyrosines, or one unmodified tyrosine within a peptide which also contained one nitrated tyrosine, where these modification were identified in the Mascot search (width 0.1 Da). One observed peptide sequence (RHPYFYAPELLYYANK) – in both its unmodified and various nitrated forms - was excluded from the data analysis, due to more than one XIC peak being generated for the m/z values of nitration at multiple tyrosine residues. With this single exception, there were no detected peptides containing more than two unmodified tyrosines or more than two nitrated tyrosines. The XIC peak area of each peptide that contained one or two nitrotyrosine residues was divided by the total peak area of the nitrotyrosine containing peptide(s) and the corresponding unmodified peptide to give the relative percentage modification. The above analysis of the data is based on the assumption that the modified and corresponding unmodified peptide behaved similarly in the MS analysis, for example in terms of ionization efficiency and detector sensitivity. Therefore, the calculated ratio of modified to unmodified forms for each peptide was an approximation [35]. Peaks with areas below 1.0×10^4 were excluded and the peak areas for the remaining peaks were recorded.

The Enzo nitrated BSA standard was also analyzed by the same method. The locations of nitrated tyrosine residues were confirmed by *de novo* sequencing using the Peak View software (AB SCIEX Version 1.0 [34]). A 3D ribbon cartoon indicating the locations of modified residues was generated using PyMol Molecular Graphics System (Schrödinger LLC).

Determination of protein concentration and protein carbonyls

The protein concentration was determined by the bicinchoninic acid (BCA) assay [36] (Thermo Scientific Cramlington, UK), and the protein carbonyl content was measured using a commercial EIA kit (BioCell Corporation Ltd, NZ).

Statistical analysis

Data sets were tested for a normal distribution using the Shapiro-Wilk's test and the appropriate parametric (two-way ANOVA) or non-parametric (Wilcoxon matched pairs and Kruskal-Wallis) tests were selected accordingly.

Results

Assay characteristics

The new ECL-based ELISA, described here, provides approximately fifty times more sensitivity than a colorimetric ELISA (**Figure 1**). The LLOQ was determined to be 0.04 nM nitrated BSA. Plasma was spiked with 1 nM nitrated BSA and diluted to 4 different concentrations for determination of linearity and recovery. The average linearity and recovery were 0.998 ± 0.001 ($n=3$) and $107 \pm 3\%$ ($n=4$) respectively (the full source data sets are reported in the Supplementary Material, Tables S1 and S2 respectively). A 1 in 5 dilution was selected for the analysis of serum samples as a balance between maximising the signal in samples which contained low concentrations of nitrotyrosine whilst only needing a low volume of sample.

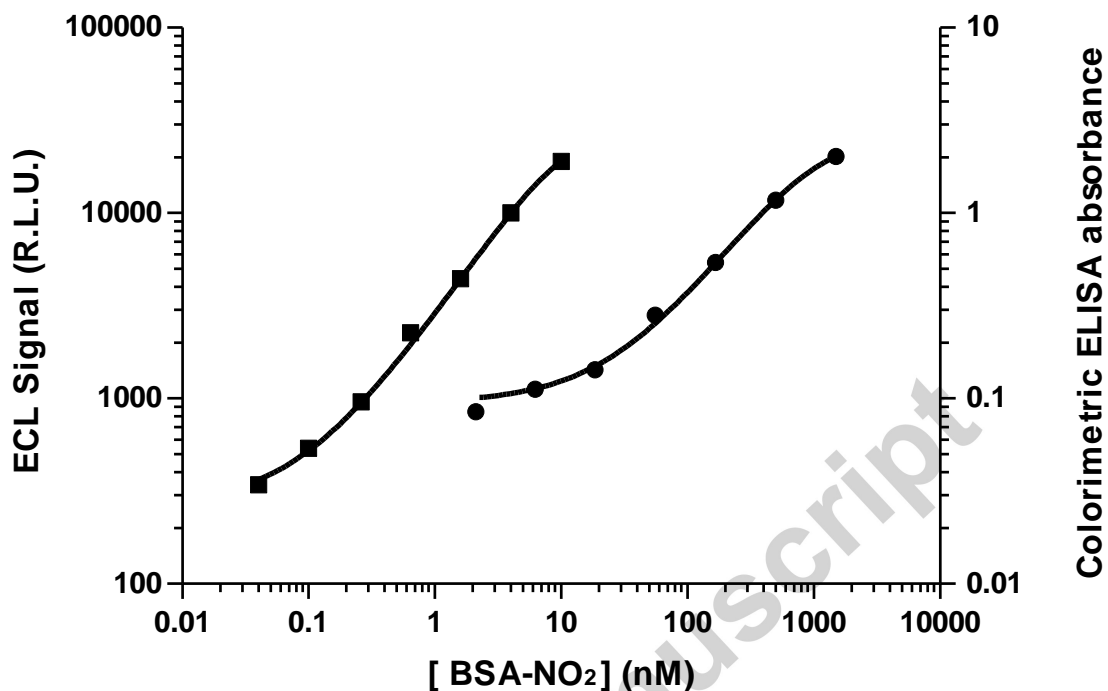


Figure 1: A comparison of typical standard curves generated by the electrochemiluminescence (ECL)-based ELISA and a commercially-available colorimetric ELISA. The limit of detection of the ECL ELISA (squares) was lower than that of the colorimetric ELISA (circles). Abbreviations, R.L.U., relative light units. BSA-NO₂, nitrated BSA.

The inter-assay coefficient of variation (CV) was determined using a control plasma sample containing a mean concentration of 2.4 nM nitrotyrosine (nitrated BSA equivalents), giving a CV of 11.3% (n=8). This CV was obtained under conditions where the assay plate was maintained under a constant temperature of 25°C, using a plate incubator (see 'Materials and Methods'). Recovery determinations were performed under conditions of either a constant temperature of 25°C, or at room temperature (without the use of the plate incubator): when four plasma samples were spiked with 10 nM nitrated BSA, the recovery of the spiked standard (mean and standard deviation (SD)) was 8.4 ± 5.4 and 8.3 ± 1.4 nM nitrotyrosine (nitrated BSA equivalents) for room temperature and 25°C, respectively. The smaller SD of the assay at a fixed 25°C indicates that this assay procedure was superior to relying on room temperature conditions.

Pre-incubation of the secondary antibody with free nitrotyrosine caused a decrease in the ELISA chemiluminescence signal compared with the signal generated in the absence of free nitrotyrosine ($p=0.03$, Kruskal-Wallis test, $n=3$). However, when free 3-nitrotyrosine was incubated with the nitrated BSA sample (i.e. free 3-nitrotyrosine and 3-nitrotyrosine residues within BSA were competing for antibody binding) there was no decrease in the signal, despite the concentration of free nitrotyrosine being in excess of the protein associated nitrotyrosine (free nitrotyrosine would be expected to inhibit the assay signal due to the 'sandwich' design needing two epitopes), suggesting that the antibody has a higher affinity for the protein associated nitrotyrosine.

The commercial nitrated BSA standard (Enzo Life Sciences) was analyzed by mass spectrometry and the three confirmed nitrotyrosine residues identified are highlighted in

Figure 2.

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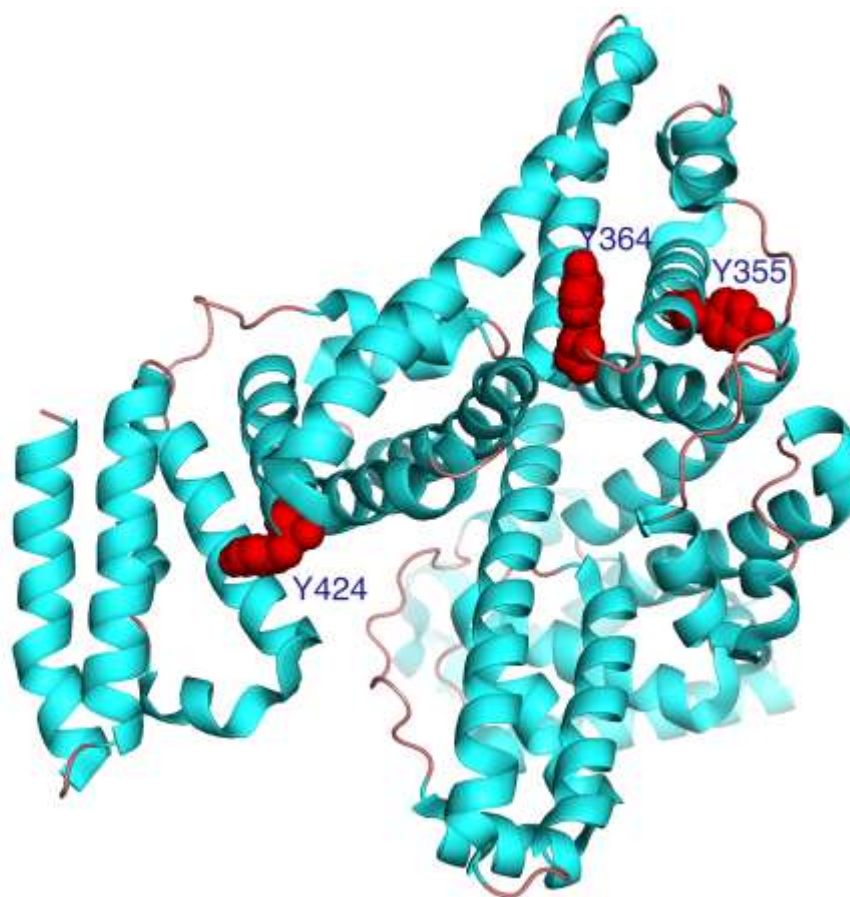


Figure 2: Ribbon cartoon representing the three dimensional structure of native bovine serum albumin (BSA, pdb 3V03), and showing the positions of the nitrated tyrosine residues in the commercial BSA standard, as identified by mass spectrometry. Tyrosine residues (Y) confirmed to be nitrated by mass spectrometry (protocol described in 'Materials and Methods' subsection 'Validation of the new ECL-based ELISA by mass spectrometry') are highlighted in red, and sequence coverage was 65%. The α -helices are shown in blue and the connecting loop regions in pink. BSA contains 21 tyrosine residues: approximately 70% of these residues are buried and 30% exposed. Of the tyrosine residues highlighted above, tyrosine 355 and tyrosine 364 are both buried and tyrosine 424 is exposed. This figure was produced using PYMOL (Schrödinger, Inc).

Validation of the ECL-based ELISA by mass spectrometry

BSA modified with peroxynitrite showed a concentration-dependent increase in nitration in both the ELISA and mass spectrometry (MS) analysis. Sequences analyzed in the MS were: K.YLYEIAR.R, R.RHPEYAVSVLLR.L, K.YICDNQDTISSK.L, K.LGEYGFQNALIVR.Y, K.DAFLGSFLYEYSR.R, K.EYEATLEECCA.K.D, K.YNGVVFQECQAEDK.G, R.MPCTEDYLSLILNR.L, R.RPCFSALTPDETYVPK.A and K.DDPHACYSTVFDK.L. The ELISA signal showed an approximate doubling in response to a doubling of peroxynitrite concentration, while the MS response showed a low increase in signal between 1.5 mM and 3.0 mM peroxynitrite. The ELISA signal for nitrotyrosine, in the modified BSA, was lowered to levels similar to that of the unmodified BSA when treated with sodium dithionite (**Figure 3**); this was also seen in the MS analysis.

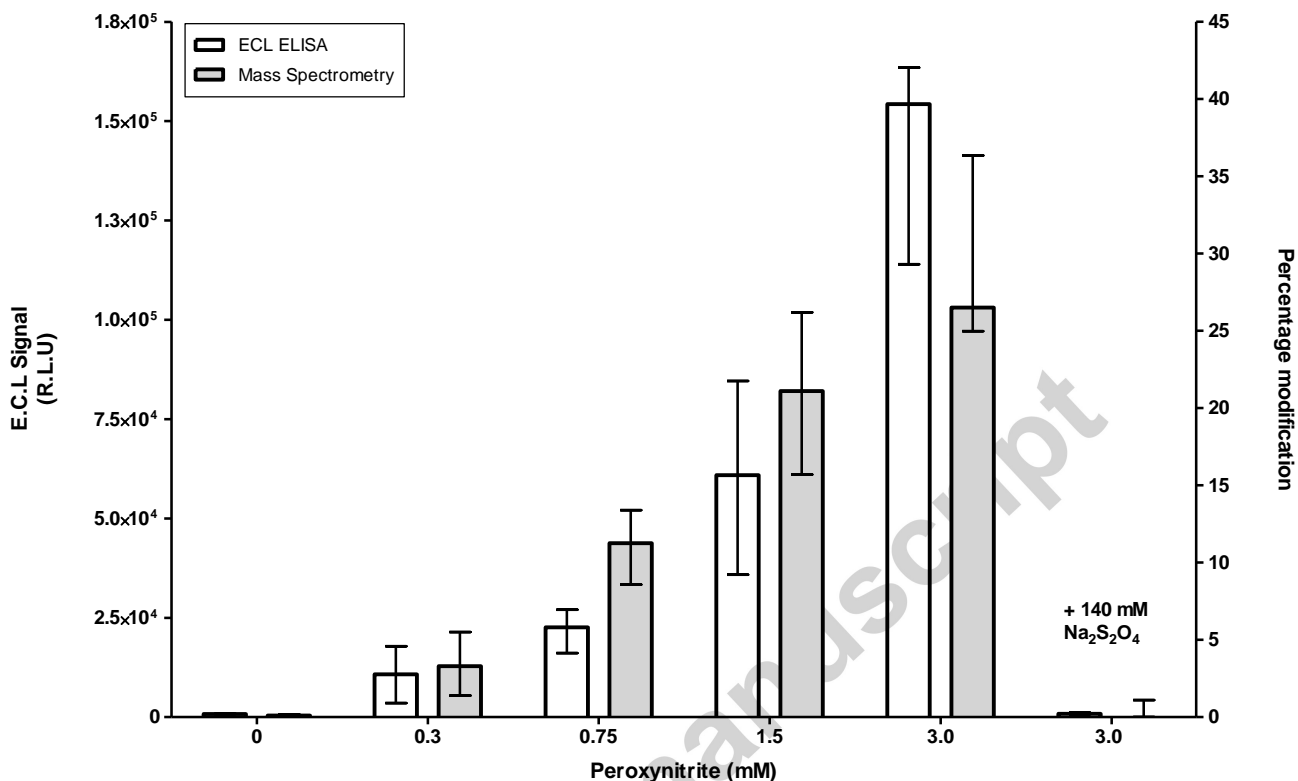


Figure 3: Extent of the nitration of native bovine serum albumin and peroxynitrite-treated bovine serum albumin, as detected by the new ECL-based ELISA in comparison with mass spectrometry. Chemically modified BSA (protocol described in Methods subsection ‘Validation of the new electrochemiluminescence-based ELISA by mass spectrometry’) was measured by the ECL-based ELISA (white bars) and by mass spectrometry (grey bars). A sample of BSA that had been modified using 3.0 mM peroxynitrite was additionally treated with Na₂S₂O₄, to reduce nitro groups to amino groups, according to the procedure described in the Methods. This is indicated on the graph as “+ 140 mM Na₂S₂O₄”. Median and interquartile ranges are shown (n=4 for each treatment). Where peptides contained more than one tyrosine residue, the signals for both mono- and di-nitrated peptides were used for calculation of the percentage modification.

Application of the assay to healthy human samples

The isolated peripheral blood mononuclear cells, and cultured U937 cells, had nitrotyrosine levels that were significantly higher than the levels observed in the plasma from healthy individuals ($p < 0.05$ and $p < 0.01$ respectively, Kruskal-Wallis test). The red blood cells also had higher levels of nitrotyrosine, compared to the plasma, but this was not statistically significant.

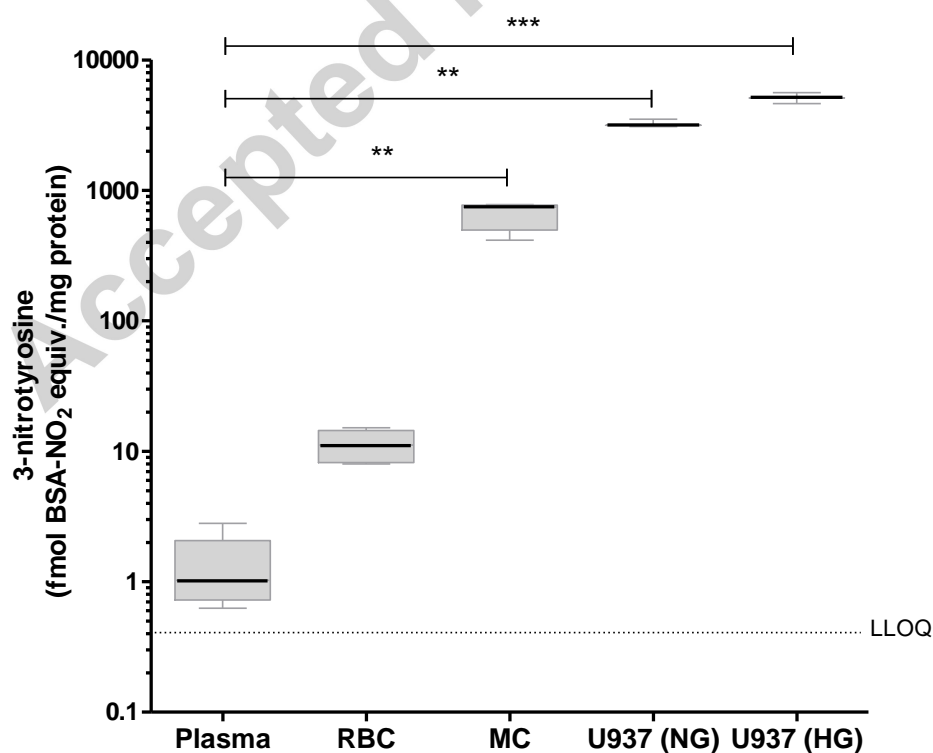


Figure 4: Comparison of 3-nitrotyrosine levels in healthy human plasma, human blood cells and a human cell line. Levels of nitration were far lower in the plasma (n=15) compared to cellular samples. 3NT was measured as per the detailed protocol in the Methods subsection 'ECL-based ELISA method'. Cell lysates were collected as per the protocol detailed in the Methods ("Cell culture" subsection). RBC (red blood cells, n=6), MC (mononuclear cells, n=4) and U937 cells (histiocytic lymphoma cell line) at a normal glucose (NG; 5.6 mM, n=3) and high glucose (HG; 24 mM, n=3) concentration. Median (long horizontal bar) and IQR (short horizontal bar) shown. A log scale has been used for the y-axis. **p<0.01, ***p<0.001 (Dunn's Multiple Comparison Test).

Application of the assay to pre- and post-surgery serum samples

There were individual variations in baseline (i.e. pre-surgery) human serum protein concentrations. A statistically significant fall in the median serum protein concentration, post-surgery, was also observed (median (IQR): 82.5 (75.7-87.6) and 73.1 (60.5-79.0) mg/mL for pre- and post-surgery serum protein concentrations, respectively (p<0.01, Wilcoxon matched pairs)). In order to take into account these sample differences in protein concentrations, the ECL-based ELISA results for nitrotyrosine were normalized to the serum protein content and expressed as fmol nitrated BSA equivalents/mg protein. A small but statistically significant (p<0.05, Wilcoxon matched pairs) increase in normalized serum nitrotyrosine was seen post-surgery (**Figure 5**): the median (IQR) values were 0.59 (0-1.3) and 0.97 (0-1.7) nitrotyrosine (fmol nitrated BSA equivalents/mg protein) for pre- and post-surgery, respectively.

As expected, in this example of a tissue insult leading to an inflammatory response, the median serum concentration of the acute phase protein, CRP, was increased significantly following surgery: the median (IQR) values were: 3.5 (0.97-6.25) and 36 (8.0-86) mg/mL before and after surgery, respectively ($p < 0.0001$, Wilcoxon matched pairs). There was no correlation between serum CRP concentrations and serum nitrotyrosine levels. There was a negative correlation between serum protein concentration and CRP concentration (Spearman's rank correlation coefficient $r_s = -0.335$, $n = 69$; $p = 0.005$).

Median serum protein carbonyls also increased significantly following surgery: the median (IQR) values were 0.30 (0.16-0.41) and 0.39 (0.17-0.52) nmol/mg protein, before and after surgery, respectively ($p < 0.05$, Wilcoxon matched-pairs test). However, serum protein carbonyl levels did not correlate significantly with nitrotyrosine levels.

For some of the samples, clinical data were available in respect of the white blood cell populations in the blood (pre-surgery $n = 26$ and post-surgery $n = 16$: 14 matched samples). A significant increase was seen in the total neutrophil number following surgery: the median (IQR) values were, 3.73 (3.37-5.46) and 8.65 (7.40-9.94) neutrophils ($\times 10^9/L$) before and after surgery, respectively ($p = 0.0006$, Wilcoxon matched-pairs test). Neutrophil number correlated positively with CRP concentration (Spearman's rank correlation coefficient, $r_s = 0.6152$, $n = 40$, $p < 0.0001$).

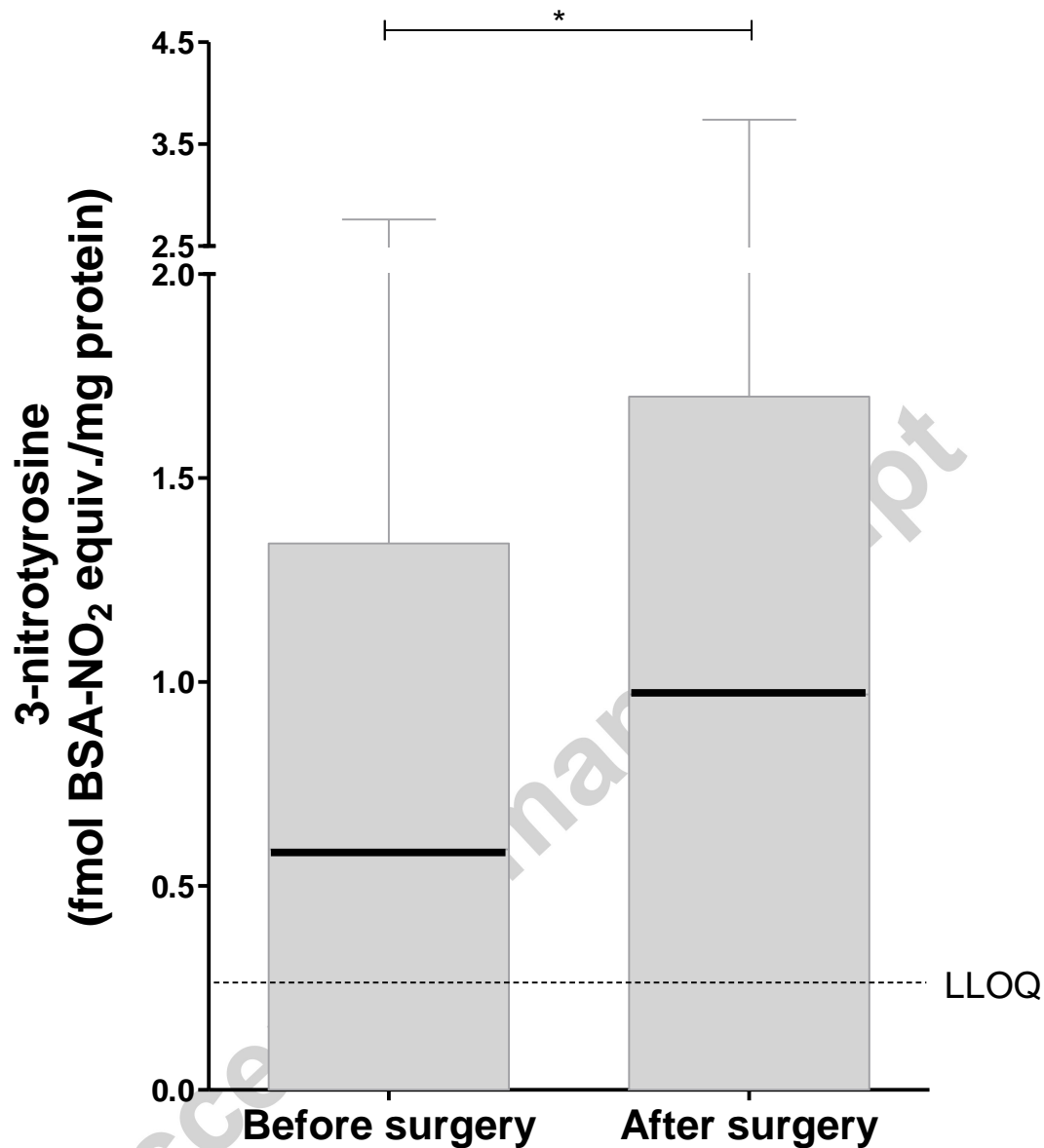


Figure 5: Median serum protein nitration levels before hospital surgery, compared with the same patients after surgery. 3NT was measured using the protocol described in the 'Materials and Methods' in paired patient serum samples, before and after surgery (n=35 in each group). Serum nitration levels increased following major elective surgery (*p=0.02, Wilcoxon matched pairs). The dashed line represents the lower limit of quantification (LLOQ). The box represents the lower and upper quartiles and the central line is the median. The whiskers show the maximum values.

Discussion

A sensitive ECL-based ELISA was developed for the measurement of nitrotyrosine in bio-fluids. This assay has an improved LLOQ compared to existing colorimetric ELISAs. The determined LLOQ of the ECL-based ELISA was 0.04 nM nitrated BSA equivalents, compared with a LLOQ of about 2 nM for many commercial kits. The new assay also has good linearity, recovery and specificity. The improved sensitivity of the present assay is crucial, since healthy human plasma samples often contain nitrotyrosine concentrations that are below the limits of detection of some commercially available spectrophotometry-based ELISAs [8, 37]. A commercial nitrated BSA standard was used in the new ECL-based assay. In order to gain a better understanding of the structural nature of the commercial nitrated BSA protein (produced by exposing BSA to tetranitromethane), characterisation by mass spectrometry was carried out. Sequence coverage was 65% and three residues were confirmed by *de novo* sequencing, to be nitrated. These residues were in the peptide sequences: ³⁴⁷DAFLGSFLY(NO₂)EYSR, ³⁶⁰RHPEY(NO₂)AVSVLLR and ⁴²¹LGEY(NO₂)GFQNALIVR, corresponding to nitrotyrosine residues at positions 355, 364 and 424, respectively. As the coverage did not include the entire polypeptide chain we cannot be sure that there was no nitration at other tyrosine residues.

The sandwich ELISA design of the present ELISA necessitates that at least two epitopes (each containing a nitrotyrosine residue) must be present for a signal to be produced by the nitrated monomer of BSA. A chemically-modified form of BSA, analyzed here by MS, was also generated in our laboratory by treatment with peroxynitrite (unlike the commercial standard which was nitrated using

tetranitromethane) and was shown to be nitrated at the same tyrosine positions as found for the commercial standard above. The tyrosine residues at positions 355 and 424 of BSA also showed a small degree of nitration in the untreated BSA, suggesting the possibility that these tyrosine residues are nitrated *in vivo*. The role of nearby charged amino acids in directing which tyrosine residues are nitrated in a protein has been previously discussed [38, 39] suggesting a nearby charge favors nitration but other studies [40] suggest uncharged polar residues are more favourable. Within the current study, all of the more highly nitrated tyrosine residues analyzed were adjacent to a negatively charged glutamic acid.

The successful use of a single monoclonal anti-nitrotyrosine antibody, as both the primary and secondary antibody within the sandwich ELISA reported here, suggests that the antibody is able to bind to at least two different epitopes containing a nitrated tyrosine residue. As the tyrosine residues 356 and 364 are close together, the simultaneous binding of two IgG molecules to these two residues is unlikely, due to steric hindrance. It may therefore be implied that residue 424 putatively defines one of the binding sites for the anti-nitrotyrosine antibody. This commercial antibody, employed in the current study, was raised against peroxynitrite-treated keyhole limpet hemocyanin but the exact epitope in this protein is unknown. Although only three nitrated tyrosine residues (355, 364 and 424) were identified by *de novo* sequencing, the sequence coverage was limited to 65%. Therefore, it is not possible to exclude the possibility that other tyrosine residues were nitrated within the BSA standard and were recognized by the antibody.

Some ELISA-based studies report nitrotyrosine levels to be undetectable in healthy human plasma [8, 37]. However, other studies have suggested levels far higher than those observed in our study, even in good health (i.e. the absence of inflammation) [41, 42]. ELISA methods utilising different antibodies may report different nitrotyrosine levels for the same sample set. This is due to antibody differences, such as whether the antibody is mono- or poly-clonal and which antigenic protein was used to raise the antibody. Different nitrated proteins have epitopes consisting of different amino acid sequences, producing varying affinities for the sequences surrounding the nitrated residues in the assayed bio-fluids. Some antibodies may also cross-react with other oxidative amino acid modifications [20, 21].

Despite a wealth of studies utilising ELISAs for nitrotyrosine measurement in clinical samples, the nitrotyrosine levels reported in the literature are hard to compare in a robust manner. For example, along with differences in the antibodies used, the ELISA design (indirect, competitive and sandwich) and the nature of the nitrotyrosine-containing standard have also varied. The report by Ter Steege et al. [8] used nitrated plasma as a standard, where others have used nitrated BSA [20, 25, 42]. Additionally, there is ambiguity in the reported results, as nitrotyrosine levels were often reported as absolute concentrations when they were in fact “standard equivalent” concentrations. In order for an absolute nitrotyrosine concentration to be reported, an essential starting point would be that the exact amount of nitrated tyrosine in the protein standard must be known. However, often all that is reported is the protein concentration of the nitrated protein standard. Therefore, the reported concentrations presumably relate to the nitrated protein concentration, not the total nitrotyrosine content.

On measurement of the relative extent of nitration of native and chemically modified BSA, we observed that there was a good agreement between the described ECL-based ELISA and mass spectrometry analysis. In both methods an increasing signal was seen with increasing peroxyntirite exposure and a decrease following treatment with sodium dithionite (known to chemically reduce nitro groups to amino groups [25]). In relation to the ELISA signal obtained from the addition of increasing concentrations of peroxyntirite, up to a maximum final concentration of 3.0 mM, a doubling of peroxyntirite concentration corresponded roughly to a doubling of ELISA response. In contrast, the MS signal intensity appeared to exhibit a disproportionately lower increase in response to doubling peroxyntirite concentrations, from 1.5 mM to 3.0 mM. The explanation for this is unclear. However, it is possible that competing chemical reactions (e.g. radical-mediated polypeptide scission) become more pronounced at higher peroxyntirite concentrations, thereby decreasing the yield of MS-detectable 3NT. Our MS analysis only included 4 modifications in order to avoid artefacts. For this reason we cannot rule out the possibility that the results obtained for the percentage modification, by tyrosine nitration, have been influenced by other unaddressed modifications, such as hydroxylation of tryptophan and tyrosine. Simultaneously, the ELISA signal exhibited a disproportionate enhancement, at higher peroxyntirite concentrations, perhaps as a result of protein aggregation and/or unfolding to provide additional epitopes for antibody binding. At intermediate treatment concentrations (0.3 mM – 1.5 mM), mass spectrometric analysis appeared to give higher nitrotyrosine levels than the ELISA; however, it has previously been reported for a synthetic peptide that the MS signal intensity of the nitro-peptide was approximately 2.5-fold higher than that of the native

peptide [43]. This would lead to an overestimation by MS of the extent of nitration, although the extent of such an effect is likely to be sequence-dependent. Tandem mass spectrometry is considered to be the 'gold-standard' method for measuring nitrotyrosine, especially free nitrotyrosine, as targeted methods such as MRM are highly accurate and can quantify the absolute nitrotyrosine concentration [22, 23, 44]. However, this accolade comes with a few important caveats when dealing with complex biological samples such as plasma/serum, owing to many factors that may confound analysis, and great variation in ratios of 3-nitrotyrosine to unmodified tyrosine has been discussed in the literature [44]. For the measurement of nitrated proteins via the generation of free 3-nitrotyrosine, samples must be processed appropriately: digestion by acid hydrolysis can increase the risk of artificial formation of 3-nitrotyrosine (due to nitrite in the sample) and steps must be taken to minimize this formation [44]. Alternatively, the protease mixture pronase from *Streptomyces griseus* can be used, but it has been reported that the incubation times required for total digestion are protein-dependent, and may be as long as 3 days [45]. Mass spectrometry is also not ideally suited to the analysis of a large number of samples (such as in a clinical setting) as it is relatively time-consuming (low-throughput) and expensive. ELISA methods can address some of these issues, and we have shown here that the ECL-based ELISA is in good agreement with the mass spectrometry with regard to the direct measurement of the relative amounts of protein-bound nitrotyrosine in the nitrated BSA samples.

Commercial ELISA kits, for measuring nitrotyrosine are unlikely to be sensitive enough to detect the low levels of nitrotyrosine present in some human serum samples, whereas the ECL-based ELISA offers fifty-fold greater sensitivity, allowing the detection

of nitrotyrosine in a greater proportion of these samples. Multiple sample types were assayed using this new ECL-based ELISA. These results showed that nitrotyrosine levels were far higher in healthy human cell-derived samples compared to the circulating plasma levels. The highest nitrotyrosine levels were observed in unstimulated U937 cells in this study. As is conventional, these cells were cultured under atmospheric oxygen concentrations (21% oxygen). However, the U937 cell line is derived from a histiocytic lymphoma. Lymphocytes circulate through the blood and lymphatic system and, within secondary lymph organs, the physiological oxygen concentration may be as low as 5% [46]. As such, the cultured cells could be considered to be in a state of oxidative stress [47]. This may explain the relatively high levels of nitrotyrosine observed in these cells.

The ECL-based ELISA was also applied to the measurement of nitrotyrosine in serum from patients undergoing major elective surgery, with samples collected before and after the surgery. This provided paired samples prior to and following an inflammatory insult (as confirmed by CRP levels and neutrophil counts). A change in oxidative stress status was confirmed by measuring serum protein carbonyl levels. A rise in oxidative stress, post-surgery, has previously been linked with tissue damage and recovery time [48, 49]. As the reactive oxygen species which have often been implicated in nitrotyrosine formation (peroxynitrite) have also been implicated in ischemia-reperfusion injury [50], protein nitration may be an important marker of damaging ROS production following surgery that involves ischemia-reperfusion, which would have been the case for some of the surgeries performed in this study.

Within this study, there was a wide variation in baseline (i.e. before surgery) serum nitration levels, with the observed median (and IQR) values for serum nitrotyrosine levels in patients being 0.048 (0-0.102) nM nitrated BSA equivalents before surgery. The large range of values may be attributed to the range of medical conditions within the sample group. Possibly due to the use of fluid supplementation during surgery and/or recovery (although the relevant clinical information was not available to allow a resolution of this possibility), there was a significant ($p=0.007$) drop in the median serum protein concentration following surgery. Therefore, it was necessary for the serum nitrotyrosine levels to be adjusted for the serum protein concentration.

Increased oxidative stress (and nitration) may also lead to an enhanced degradation of modified proteins. The 20S proteasome is largely responsible for the degradation of proteins which have been modified as a result of oxidative stress [51, 52] and peroxynitrite-modified proteins are degraded faster than unmodified proteins [53, 54]. When Souza et al. [54] exposed Cu-Zn superoxide dismutase to peroxynitrite, nitration of a single tyrosine residue was determined to be the only structural modification, and degradation by the proteasome was enhanced. This suggests that nitrated proteins are marked for degradation and therefore more likely to be removed than non-nitrated proteins. Such a process may partially explain why the concentrations of protein bound nitrotyrosine in some of the clinical samples analyzed in the present study, were very low. However, it should be noted that when oxidative stress becomes excessive proteins may cross-link and aggregate, preventing them from being degraded and may even bind to, and inhibit, the proteasome [52, 55]. Therefore, in diseases with excessive oxidative stress, the levels of protein-associated nitrotyrosine will not be affected by

protein degradation. However, this might affect analysis by mass spectrometry and recognition of nitrotyrosine by antibodies.

Radák et al. [56] measured serum nitrotyrosine daily in people doing a super-marathon; this extreme exercise induces an inflammatory reaction. The authors noted an increase in serum nitrotyrosine, when comparing the baseline with day one of the race, but then the levels of nitrotyrosine plateaued, despite the increasing intensity of the race. Radák et al. [56] proposed that this was due to the processes of nitration and degradation reaching equilibrium. However, the authors did not mention measuring the protein concentration of the serum over the course of the race (intense exercise can cause proteinuria), or correction of their results for protein content. If a drop in protein concentration occurred, each day, then an adjustment for protein concentration would have shown nitration levels increasing rather than remaining constant. However, few published studies show nitrotyrosine results corrected for protein concentrations and this may account for some of the discrepancies between different studies. Weber et al. [25] suggested that all samples should be diluted to the same protein concentration prior to measurement. This was highlighted as a methodological issue (as protein concentration may affect assay performance) but would also act as a way of normalizing the results from different samples to a single protein concentration.

Limitations

Some limitations of this study should be noted. Firstly, a validation of the novel ELISA against the “gold standard” MRM-based method for analysis of free nitrotyrosine was not carried out on the patient samples, owing to the complexity of the sample

preparation for the mass spectrometry method. This would be desirable for future work to provide additional confirmation of the levels of nitrotyrosine in individual samples. The patient sample analysis was also limited by the large variety of presenting diseases in the studied patient population, as well as the range in the types of surgery to which the patients were subjected. Additionally, the numbers for any one type of surgery were too small to allow a statistical analysis of whether a particular surgery type was associated with significantly higher levels of nitrotyrosine. The blood cell populations were only harvested from healthy volunteers. Therefore, there is currently no information regarding the effects of surgery/inflammation on the levels of nitrotyrosine in different human blood cell types. Further studies with highly defined disease groups, but with large sample numbers for each group, along with the collection of both serum/plasma and blood cells, would address these issues.

Although work has already been published on the low cross-reactivity of the anti-nitrotyrosine antibody to various other nitro- compounds [30], cross-reactivity towards peptides or proteins containing nitrotryptophan was not tested and cross-reactivity cannot be ruled out.

Conclusion

Notwithstanding these limitations, the ECL-based ELISA described here was suitable for nitrotyrosine-based determination of oxidative stress in healthy human plasma, primary and cultured cell populations, and serum pre- and post-surgery study, and provides improved sensitivity as well as a robust and cost-effective method to measure nitrotyrosine in clinical samples. Even with the improved sensitivity of the ECL-based

ELISA, some patient serum samples were below the LLOQ. However, the assay still provided a method by which a statistically significant increase in serum nitrotyrosine could be detected *in vivo* following activation of an inflammatory response (induced by surgery). There is room for further improvement of the present assay, such as validation against MRM analysis of free nitrotyrosine and the reporting of results as an absolute nitrotyrosine concentration rather than as nitrated BSA equivalents. The latter is a weakness found in many reported ELISA results and needs to be addressed in future work, in order to facilitate the comparison of results from different laboratories.

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References

- [1] S. Bartesaghi, G. Ferrer-Sueta, G. Peluffo, V. Valez, H. Zhang, B. Kalyanaraman, R. Radi, Protein tyrosine nitration in hydrophilic and hydrophobic environments, *Amino Acids* 32(4) (2007) 501-15.
- [2] J.M. Souza, G. Peluffo, R. Radi, Protein tyrosine nitration—Functional alteration or just a biomarker?, *Free Radical Biology & Medicine* 45(4) (2008) 357-366.
- [3] D.M. Kuhn, S.A. Sakowski, M. Sadidi, T.J. Geddes, Nitrotyrosine as a marker for peroxynitrite-induced neurotoxicity: The beginning or the end of the end of dopamine neurons?, *Journal of Neurochemistry* 89(3) (2004) 529-536.
- [4] E.R. Frears, Z. Zhang, D.R. Blake, J.P. O'Connell, P.G. Winyard, Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite, *FEBS Lett* 381(1-2) (1996) 21-4.
- [5] J.P. Gaut, J. Byun, H.D. Tran, W.M. Lauber, J.A. Carroll, R.S. Hotchkiss, A. Belaouaj, J.W. Heinecke, Myeloperoxidase produces nitrating oxidants in vivo, *The Journal of Clinical Investigation* 109(10) (2002) 1311-1319.
- [6] L.K. Stamp, I. Khalilova, J.M. Tarr, R. Senthilmohan, R. Turner, R.C. Haigh, P.G. Winyard, A.J. Kettle, Myeloperoxidase and oxidative stress in rheumatoid arthritis, *Rheumatology (Oxford, England)* 51(10) (2012) 1796-803.
- [7] F. Khan, A.A. Siddiqui, R. Ali, Measurement and Significance of 3-Nitrotyrosine in Systemic Lupus Erythematosus, *Scandinavian Journal of Immunology* 64(5) (2006) 507-514.
- [8] J.C.A. Ter Steege, L. Koster-Kamphuis, E.A. van Straaten, P.P. Forget, W.A. Buurman, Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA, *Free Radical Biology & Medicine* 25(8) (1998) 953-963.
- [9] A. Nissim, P.G. Winyard, V. Corrigan, R. Fatah, D. Perrett, G. Panayi, Y. Chernajovsky, Generation of neoantigenic epitopes after posttranslational modification of type II collagen by factors present within the inflamed joint, *Arthritis & Rheumatism* 52(12) (2005) 3829-3838.
- [10] M.H. Shishehbor, R.J. Aviles, M.L. Brennan, X. Fu, M. Goormastic, G.L. Pearce, N. Gokce, J.F. Keaney, Jr., M.S. Penn, D.L. Sprecher, J.A. Vita, S.L. Hazen, Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy, *Jama* 289(13) (2003) 1675-80.
- [11] E. Eleuteri, A. Di Stefano, F.L.M. Ricciardolo, F. Magno, I. Gnemmi, M. Colombo, R. Anzalone, F. Cappello, G. La Rocca, F.T. Genta, G. Zummo, P. Giannuzzi, Increased nitrotyrosine plasma levels in relation to systemic markers of inflammation and myeloperoxidase in chronic heart failure, *International Journal of Cardiology* 135(3) (2009) 386-390.
- [12] U. Hink, M. Oelze, P. Kolb, M. Bachschmid, M.-H. Zou, A. Daiber, H. Mollnau, M. August, S. Baldus, N. Tsimingias, U. Walter, V. Ullrich, T. Münzel, Role for peroxynitrite in the inhibition of prostacyclin synthase in nitrate tolerance, *Journal of the American College of Cardiology* 42(10) (2003) 1826-1834.
- [13] T. Munzel, A. Daiber, S. Steven, L.P. Tran, E. Ullmann, S. Kossmann, F.P. Schmidt, M. Oelze, N. Xia, H. Li, A. Pinto, P. Wild, K. Pies, E.R. Schmidt, S. Rapp, S. Kroller-Schon, Effects of noise on vascular function, oxidative stress, and inflammation: mechanistic insight from studies in mice, *Eur Heart J* 38(37) (2017) 2838-2849.

- [14] H. Kaur, B. Halliwell, Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients, *FEBS Letters* 350(1) (1994) 9-9.
- [15] M. Ohya, S. Marukawa, T. Inoue, N. Ueno, K. Hosohara, N. Terada, H. Kosaka, Plasma nitrotyrosine concentration relates to prognosis in human septic shock, *Shock* 18(2) (2002) 116-116.
- [16] G. Wang, S.S. Pierangeli, E. Papalardo, G. Ansari, M.F. Khan, Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity, *Arthritis & Rheumatism* 62(7) (2010) 2064-2072.
- [17] M. Pirro, G. Schillaci, M.R. Mannarino, G. Savarese, G. Vaudo, D. Siepi, R. Paltriccia, E. Mannarino, Effects of rosuvastatin on 3-nitrotyrosine and aortic stiffness in hypercholesterolemia, *Nutrition, Metabolism and Cardiovascular Diseases* 17(6) (2007) 436-441.
- [18] J. Frijhoff, P.G. Winyard, N. Zarkovic, S. Davies, R. Stocker, D. Cheng, A. Knight, E.L. Taylor, J. Oettrich, T. Ruskovska, A. Cipak Gasparovic, A. Cuadrado, D. Weber, H.E. Poulsen, T. Grune, H.H. Schmidt, P. Ghezzi, Clinical relevance of biomarkers of oxidative stress, *Antioxidants & Redox Signaling* 23(14) (2015) 1144–1170.
- [19] D. Yi, B.A. Ingelse, M.W. Duncan, G.A. Smythe, Quantification of 3-nitrotyrosine in biological tissues and fluids: generating valid results by eliminating artifactual formation, *Journal of the American Society for Mass Spectrometry* 11(6) (2000) 578-586.
- [20] J. Khan, D.M. Brennand, N. Bradley, B. Gao, R. Bruckdorfer, M. Jacobs, D.M. Brennan, 3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method, *Biochemical Journal* 330(Pt 2) (1998) 795-795.
- [21] T. Franze, M.G. Weller, R. Niessner, U. Poschl, Comparison of nitrotyrosine antibodies and development of immunoassays for the detection of nitrated proteins, *The Analyst* 129(7) (2004) 589-96.
- [22] H. Ryberg, K. Caidahl, Chromatographic and mass spectrometric methods for quantitative determination of 3-nitrotyrosine in biological samples and their application to human samples, *Journal of Chromatography B* 851(1–2) (2007) 160-171.
- [23] D. Tsikas, K. Caidahl, Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma, *Journal of Chromatography. B, Analytical technologies in the biomedical and life sciences* 814(1) (2005) 1-9.
- [24] Y.C. Sun, P.Y. Chang, K.C. Tsao, T.L. Wu, C.F. Sun, L.L. Wu, J.T. Wu, Establishment of a sandwich ELISA using commercial antibody for plasma or serum 3-nitrotyrosine (3NT). Elevation in inflammatory diseases and complementary between 3NT and myeloperoxidase, *Clinica Chimica Acta* 378(1) (2007) 175-180.
- [25] D. Weber, N. Kneschke, S. Grimm, I. Bergheim, N. Breusing, T. Grune, Rapid and sensitive determination of protein-nitrotyrosine by ELISA: Application to human plasma, *Free Radical Research* 46(3) (2012) 276-285.
- [26] MSD, Electrochemiluminescence.
<https://www.mesoscale.com/en/technical_resources/our_technology/ecl>, 2017 (accessed June 2017.).
- [27] C.A. Shaw, E.L. Taylor, S. Fox, I.L. Megson, A.G. Rossi, Differential susceptibility to nitric oxide-evoked apoptosis in human inflammatory cells, *Free Radical Biology & Medicine* 50(1) (2011) 93-101.

- [28] C. Sundström, K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), *International Journal of Cancer* 17(5) (1976) 565-577.
- [29] A. Shrivastava, V. Gupta, Methods for the determination of limit of detection and limit of quantitation of the analytical methods, *Chronicles of Young Scientists* 2(1) (2011) 21-25.
- [30] T. Franze, M.G. Weller, R. Niessner, U. Poschl, Enzyme immunoassays for the investigation of protein nitration by air pollutants, *The Analyst* 128(7) (2003) 824-831.
- [31] J.S. Beckman, J. Chen, H. Ischiropoulos, J.P. Crow, Oxidative chemistry of peroxynitrite, *Methods in Enzymology* 233 (1994) 229-240.
- [32] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nature Protocols* 1(6) (2006) 2856-60.
- [33] MatrixScience, Matrix Science - Mascot - Peptide Mass Fingerprint, http://www.matrixscience.com/help/interpretation_help.html, 2014.
- [34] ABSCIEX, PeakView® Software | Qualitative Review | AB SCIEX.
- [35] L. Rezende Valim, J.A. Davies, K. Tveen Jensen, R. Guo, K.R. Willison, C.M. Spickett, A.R. Pitt, D.R. Klug, Identification and Relative Quantification of Tyrosine Nitration in a Model Peptide Using Two-Dimensional Infrared Spectroscopy, *The Journal of Physical Chemistry B* 118(45) (2014) 12855-12864.
- [36] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, *Analytical Biochemistry* 150(1) (1985) 76-85.
- [37] A. Ceriello, F. Mercuri, L. Quagliaro, R. Assaloni, E. Motz, L. Tonutti, C. Taboga, Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress, *Diabetologia* 44(7) (2001) 834-838.
- [38] J.M. Souza, E. Daikhin, M. Yudkoff, C.S. Raman, H. Ischiropoulos, Factors determining the selectivity of protein tyrosine nitration, *Archives of Biochemistry and Biophysics* 371(2) (1999) 169-178.
- [39] A.S. Bayden, V.A. Yakovlev, P.R. Graves, R.B. Mikkelsen, G.E. Kellogg, Factors influencing protein tyrosine nitration – structure-based predictive models, *Free Radical Biology & Medicine* 50(6) (2011) 749-762.
- [40] K.W. Seeley, S.M. Stevens, Jr., Investigation of local primary structure effects on peroxynitrite-mediated tyrosine nitration using targeted mass spectrometry, *J Proteomics* 75(6) (2012) 1691-700.
- [41] P. Rossner Jr, V. Svecova, A. Milcova, Z. Lnenickova, I. Solansky, R.M. Santella, R.J. Sram, Oxidative and nitrosative stress markers in bus drivers, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 617(1) (2007) 23-32.
- [42] S. Bo, R. Gambino, S. Guidi, B. Silli, L. Gentile, M. Cassader, G.F. Pagano, Plasma nitrotyrosine levels, antioxidant vitamins and hyperglycaemia, *Diabetic medicine* 22(9) (2005) 1185-1189.
- [43] K. Tveen Jensen, A. Reis, L. Mouls, A.R. Pitt, C.M. Spickett, Reporter ion-based mass spectrometry approaches for the detection of non-enzymatic protein modifications in biological samples, *Journal of Proteomics* 92 (2013) 71-79.

- [44] D. Tsikas, M.W. Duncan, Mass spectrometry and 3-nitrotyrosine: Strategies, controversies, and our current perspective, *Mass Spectrometry Reviews* 33(4) (2014) 237-276.
- [45] A. Daiber, M. Bachschmid, J.S. Beckman, T. Munzel, V. Ullrich, The impact of metal catalysis on protein tyrosine nitration by peroxynitrite, *Biochem Biophys Res Commun* 317(3) (2004) 873-81.
- [46] A. Ikejiri, S. Nagai, N. Goda, Y. Kurebayashi, M. Osada-Oka, K. Takubo, T. Suda, S. Koyasu, Dynamic regulation of Th17 differentiation by oxygen concentrations, *International Immunology* 24(3) (2011) 137-146.
- [47] B. Halliwell, Oxidative stress in cell culture: an under-appreciated problem?, *FEBS Letters* 540(1-3) (2003) 3-6.
- [48] K.A. Kaminski, T.A. Bonda, J. Korecki, W.J. Musial, Oxidative stress and neutrophil activation—the two keystones of ischemia/reperfusion injury, *International Journal of Cardiology* 86(1) (2002) 41-59.
- [49] F. Rosenfeldt, M. Wilson, G. Lee, C. Kure, R. Ou, L. Braun, J. de Haan, Oxidative stress in surgery in an ageing population: Pathophysiology and therapy, *Experimental Gerontology* 48(1) (2013) 45-54.
- [50] J.L. Zweier, M.A.H. Talukder, The role of oxidants and free radicals in reperfusion injury, *Cardiovascular Research* 70(2) (2006) 181-190.
- [51] T. Jung, A. Höhn, T. Grune, The proteasome and the degradation of oxidized proteins: Part II – protein oxidation and proteasomal degradation, *Redox Biology* 2(0) (2014) 99-104.
- [52] K.J.A. Davies, Degradation of oxidized proteins by the 20S proteasome, *Biochimie* 83(3-4) (2001) 301-310.
- [53] A.J. Gow, D. Duran, S. Malcolm, H. Ischiropoulos, Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation, *FEBS Letters* 385(1-2) (1996) 63-6.
- [54] J.M. Souza, I. Choi, Q. Chen, M. Weisse, E. Daikhin, M. Yudkoff, M. Obin, J. Ara, J. Horwitz, H. Ischiropoulos, Proteolytic Degradation of Tyrosine Nitrated Proteins, *Archives of Biochemistry and Biophysics* 380(2) (2000) 360-366.
- [55] R. Shringarpure, K.J.A. Davies, Protein turnover by the proteasome in aging and disease, *Free Radical Biology & Medicine* 32(11) (2002) 1084-1089.
- [56] Z. Radák, H. Ogonovszky, J. Dubecz, G. Pavlik, M. Sasvari, J. Pucsok, I. Berkes, T. Csont, P. Ferdinandy, Super-marathon race increases serum and urinary nitrotyrosine and carbonyl levels, *European Journal of Clinical Investigation* 33(8) (2003) 726-730.