Novel Immunohistochemical Technique to Quantify Nuclear Translocation of the Redox-Sensitive Transcription Factor Nrf2 Following Middle Cerebral Artery Occlusion In the Mouse

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Introduction

Cerebral ischaemia initiates numerous cellular processes that can lead to oedema and loss of viable tissue or repair and recovery. The redox-sensitive nuclear factor NF E2-Related Factor 2 (Nrf2) moves from the cytoplasm to the nucleus following experimental stroke, leading to the induction of antioxidant enzymes such as hemoxygenase-1 (HO-1). Notably, Nrf2 knockout mice are affected much more severely by ischaemia-reperfusion injury (Wang *et al.,* 2007) while over-expressing Nrf2 by injecting anti-oxidants such as plumbagin (Son *et al.,* 2010) in a mouse model of stroke.

The aim of the present study was to develop a technique to quantify Nrf2 and its target protein HO-1 in tissue sections and to use this to demonstrate changes in intracellular distribution of the transcription factor Nrf2 in brains from mice that had undergone experimental focal ischaemia-reperfusion using the middle cerebral artery occlusion model of stroke.

Methods

Ischaemia-reperfusion model

Mice (C57Bl/6: 25-30 g) were subjected to 45 min middle cerebral artery occlusion (MCAO) under isoflurane anesthesia (2% initial, 1% to 1.5% maintenance in 30% O_2 and 70% N_2) and allowed to recover for 24 hours before they were killed.

Tissue preparation

The brains were flushed, fixed with formaldehyde, embedded in paraffin wax and 6 µm sections were obtained. Sections were processed to remove the wax and retrieve epitopes before treatment with a polyclonal anti-Nrf2 antibody, followed by a biotinylated secondary antibody and streptavidin-horseradish peroxidase (HRP) conjugate. Propidium iodide (PI) was used to identify nuclei.

Quantitative analysis of Nrf2

Sequential images of diaminobenzidine reaction product (DAB) development formed in the presence of hydrogen peroxide under a Nikon Diaphot microscope were obtained at 1 s intervals. Images were captured in a computer by using a CCD camera (CoolView, Photonic Science, Surrey) and analysed using ImageHopper software (Samsara Research, Surrey).

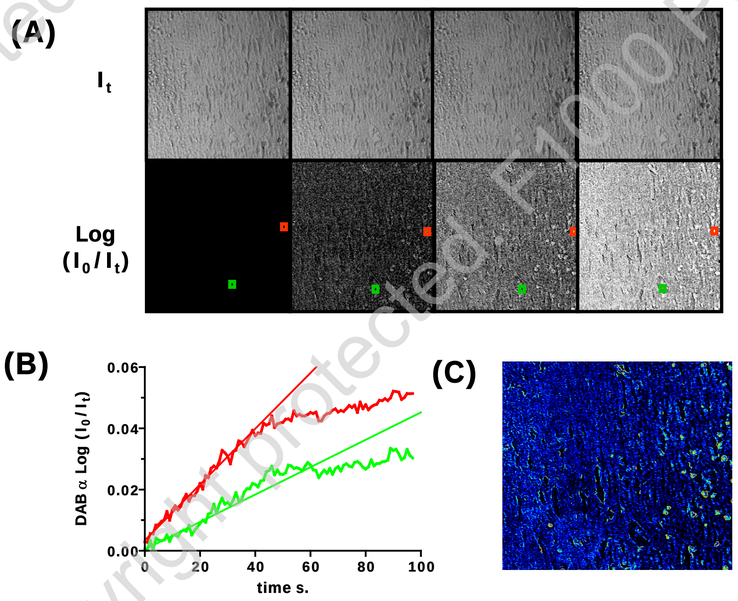

Fig. 1. Image processing. (A) Sequential images were processed to give pixel values that are proportional (by Beer-Lambert) to the DAB concentration. (B) The initial rate of DAB development is proportional to the enzyme (HRP) activity. The graph shows the rates for the 2 marked spots. (C) This initial rate was applied to the image stack as a whole, and a colour palette applied. These images were used to form Fig 3 F.

The activity of HRP is proportional to the initial rate of DAB reaction product development. The fundamental assumption that underlies the technique is that the 1º and 2º antibodies are linearly related to the antigen of interest. This hypothesis was tested by dissolving known concentrations of albumin (BSA) in gelatine solution, allowing it to set and then processing as if it were a tissue.

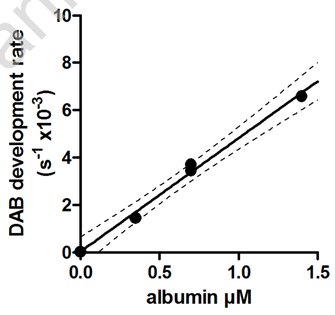

Fig 2. Proof of principle. DAB development rate shows a dose-response relationship between concentration of bovine serum albumin (BSA). This suggests that the initial rate of DAB development is directly proportional to the concentration of protein within the tissue

Results

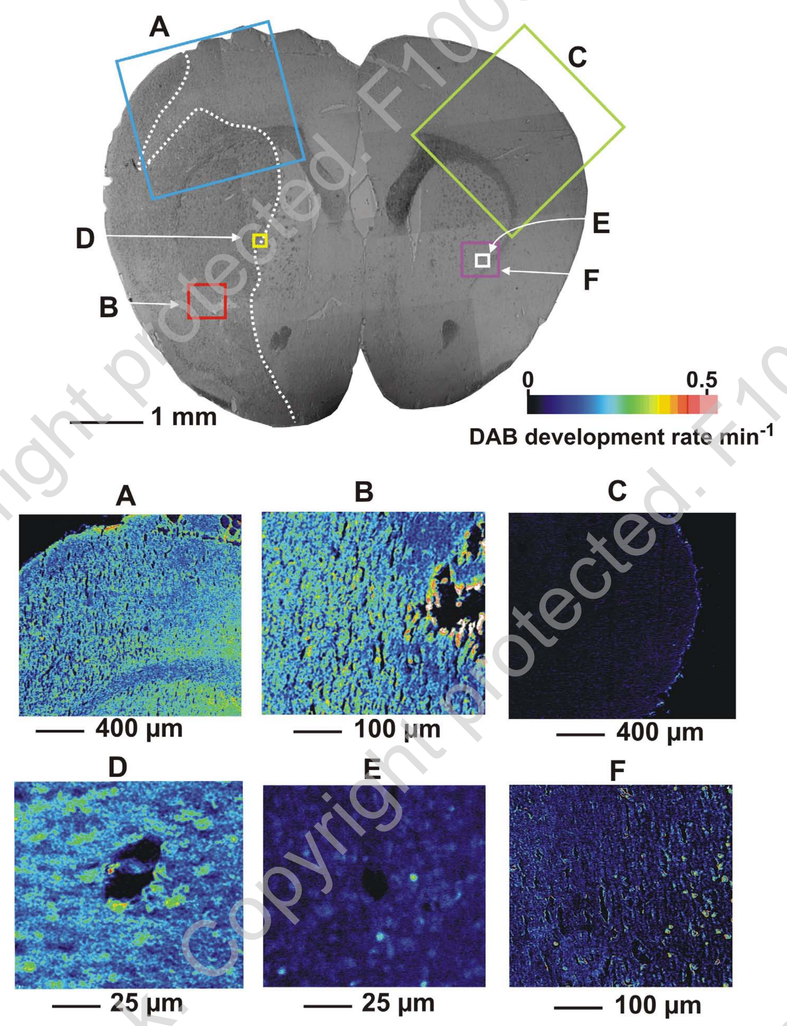

Fig 3. Nrf2 expression in infarct and control brain regions. A composite image of a coronal section before reaction with DAB-H_2O_2. The dotted line indicates the infarcted region. **A**, **B** & **D** are regions within the infarct zone, **E** & **F** are from the contr000alateral hemisphere. **C** shows a negative control section that lacked the 1º antibody. **A** is from the same section as shown in the composite, while the others are from the same brain in nearby sections.

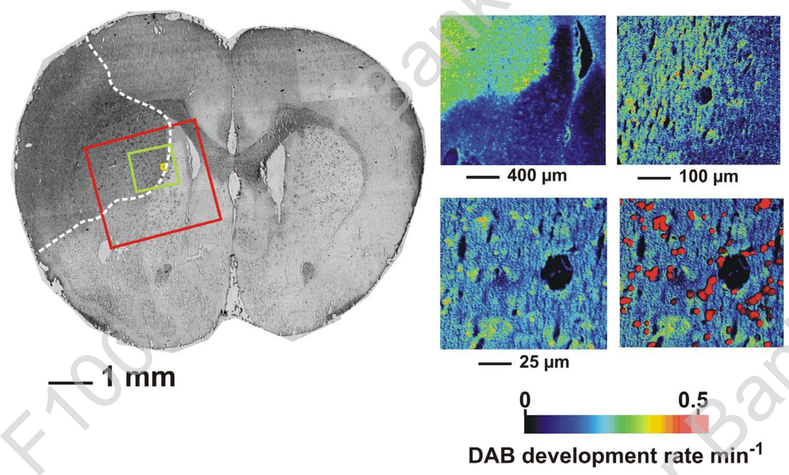

Fig 4. HO-1 expression in a sections straddling an infarct zone. A composite image of a coronal section before reaction with DAB-H_2O_2. and the low power image was from the series that made the composite, while the higher power images were obtained from neighbouring sections. The bottom right panel shows the propidium iodide image superimposed to denote the location of nuclei.

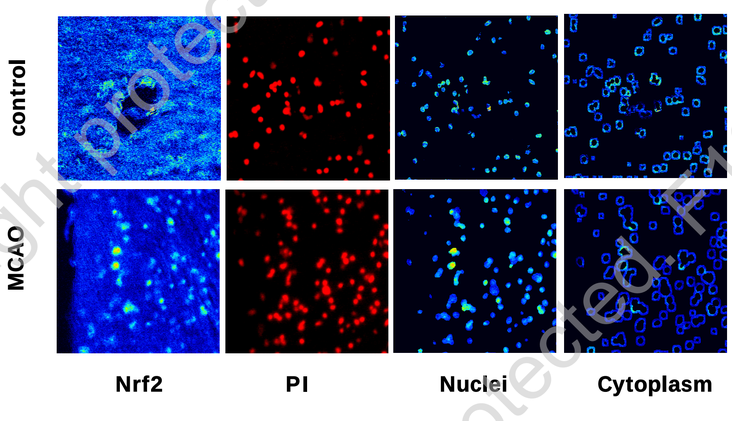

Fig 5. Nrf2 separation into nuclear and cytoplasmic compartments. Propidium iodide was used to identify nuclei. This image was converted into a mask to isolate nuclear Nrf2. The cytoplasm was defined as an annulus 5 µm wide around the nucleus. Blob counting was then used to estimate the nuclear and cytoplasmic fractions

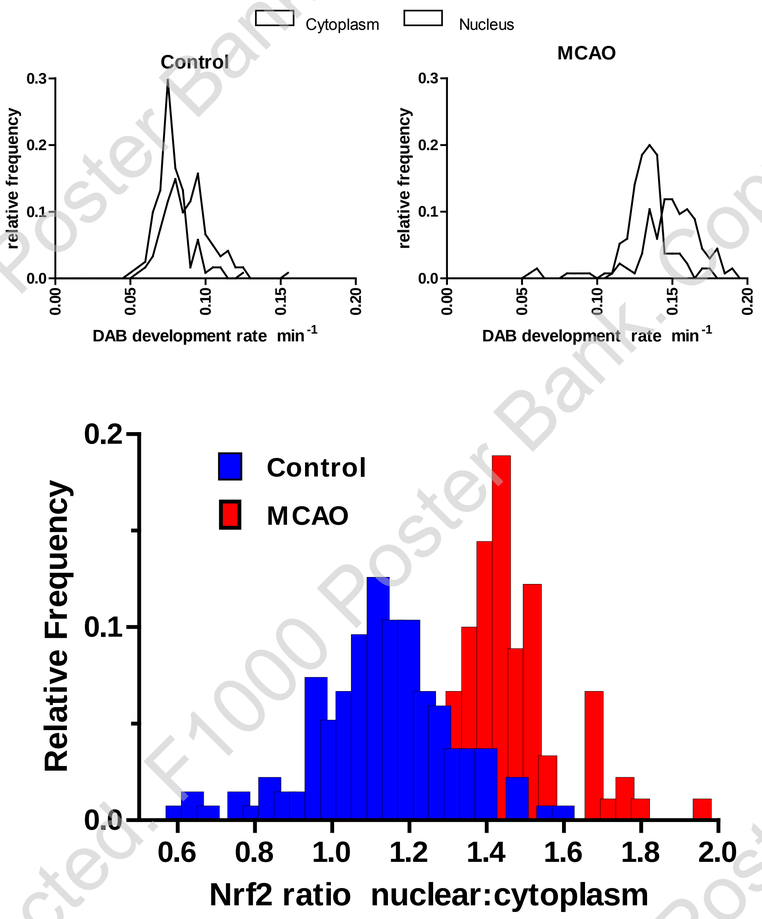

Fig 6. Analysis of Nrf2 distribution. A total of 135 nuclei were analysed from control regions in 9 sections from 5 mice and 121 nuclei from 8 sections in infarcted regions in the same mice. The frequency distributions of the nuclei and the surrounding cytoplasm show that the Nrf2 content from both increased in the infarcted regions, with a suspicion that it was greater in the nucleus. One advantage of this technique is that it is possible compare each nucleus with its surrounding cytoplasm. This ratio did increase from 1.13 ± 0.178 (mean ± sd) in the control to 1.44 ± 0.155 in the MCAO (P<0.001, 't' test)

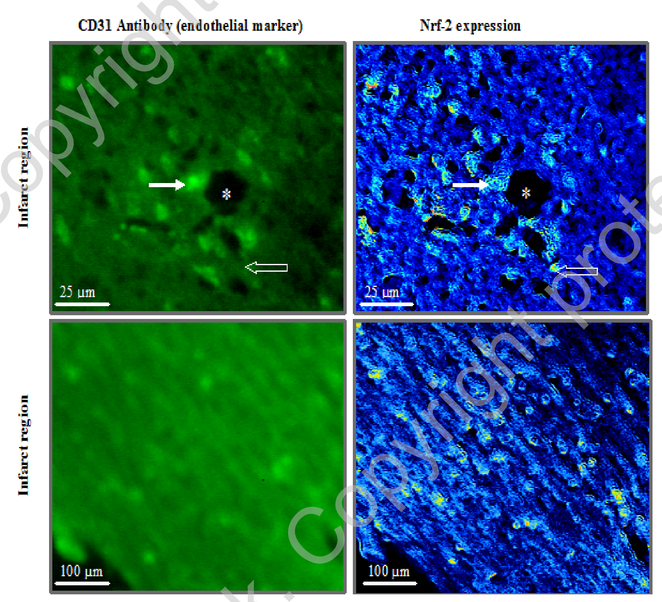

Fig 7. Localisation by cell type. Fluorescence immunohistochemistry can be performed to identify different cell types. Here is such an example using anti-CD31 with Alex Fluor labelled 2º antibody to identify endothelial cells before carrying out the DAB reaction to quantify Nrf2. This shows that there is some Nrf2 activity endothelial cell types (white arrow) close to a vessel lumen (*).

Conclusion

We have used a novel immunohistochemical technique to quantitatively analyse nuclear translocation of the redox-sensitive transcription factor Nrf2 following MCAO in the mouse. This method has merit in its ability to quantify protein expression using initial rate of DAB formation and localise protein within the tissue.

References

Son TG., et al. J Neurochem. 2010 112:1316-1326

Wang J., et al. Free Radic Biol Med. 2007 43:408-414