In vivo demonstration of Congo Red labeled amyloid plaques via perfusion in the Alzheimer disease rat model

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Abstract

Congo Red (CR) has been used for its binding affinity to amyloid fibrils for the better part of a century. Recently, our laboratory has demonstrated its ability to bind to tau protein as well. Here, we describe a novel method for fast, thorough, whole-brain labeling of amyloid plaques with CR via perfusion. We tested five different procedures which altered the volume of CR, the speed of perfusion, and the solution CR was solubilized in to determine the best results. We determined that intra-cardiac perfusion of animals with 0.5% CR in 100 ml of 50% ethanol or perfusion with 0.5 of CR in 100 ml of 10% neutral buffer formalin both perfused at a rate of 30 ml/min for 3.3 minutes resulted in the clearest CR labeling, with little background noise. The results of these methods were compatible with subsequent immunolabeling procedures for NU-1, Ferritin, and GFAP. Compared to traditional CR plaque labeling methodology, this new method allows for quick whole brain CR-labeling. This reduces the amount of time from days to mere minutes. It also reduces the variability caused by staining slides in batches. Thus, CR-perfusion is a rapid, thorough method that can be utilized to rapidly stain amyloid in the rodent brain. Further study should be done to further explore whether CR can be used to detect amyloid plaques using non-invasive approach such as MRI.

Introduction

Background

The current study is a follow-up to our recent publication (Sarkar, Raymick et al. 2020) in which we assessed the feasibility of a novel CR-staining methodology to label plaques and tangles both in rodent and human brains to save time and materials. The methodology described here involves directly introducing CR via trans-cardial perfusion in various formulations. Perfusion with all five of our unique formulations resulted in rapid, whole-brain staining of beta-amyloid via CR binding. Additionally, our results suggest that this methodology is also compatible with subsequent multiple-labeling procedures and has excellent co-localization with other traditional methods to stain for plaque load such as the NU-1 antibody. We assessed five unique formulations of CR solution to determine the best methodology for plaque staining intensity. To our knowledge, this is the first such study to describe this technique for rapid CR staining in the rodent brain.

Table 1. , below, offers a breakdown of the methods and chemicals used

Variant	Solvent	Perfusate volume (mL)	Perfusion Rate	Perfusion Time	pH
1	50% EtOH	25	10 mL/min	2.5 min	9.03
2	50% EtOH	60	20 mL/min	3.0 min	9.03
3	50% EtOH	100	30 mL/min	3.3 min	9.03
4	Heparinized Saline	60	20 mL/min	3.0	8.87
5	10% NBF	100	30 mL/min	3.3 min	7.39

Materials and Methods

Methods:

Animals: Sixteen 16-18-month-old male TgF344-AD transgenic rats were obtained from our onsite colony at the National Center for Toxicological Research (NCTR). TgF344-AD rats express human amyloid precursor protein (APP) with the Swedish mutation and human presenilin 1(PSEN1). All animal work was approved by the NCTR Institutional Animal Care and Use Committee (IACUC).

Congo Red formulations

We prepared five unique CR solutions for perfusion. We also varied the amount and rate of the perfusate. This allowed an evaluation of the five different CR solutions for there ability to "stain" different AD related pathologies. to evaluate the method that resulted in the most effective staining results. Briefly, five solutions of CR in ETOH, heparinized saline, or NBF were perfused at 10, 20, or 30 mL/min as shown in Table 1. For variant 5, 50 µL of ETOH was also added to aid in solubilizing CR.

Perfusion and Brain Preparations

Rats received an overdose of Euthasol (100mg/kg; Vibrac AH, Inc., USA) prior to perfusion procedures. Once fully anesthetized as indicated by no response to a toe pinch, animals were trans-cardially perfused with heparinized saline, followed by 10% neutral buffer formalin (NBF), then one of five unique CR formulations described in further detail in the next section.

Immunofluorescence : To evaluate the compatibility of our novel method with traditional immunofluorescence methodology was employed as shown in the table 2.

Table 2. , below, offers a breakdown of the immunofluorescence methods and chemicals used

Name of the primary antibody	Source and dilution of the primary antibody	Secondary antibody	Source and dilution of the secondary antibody	Streptavidin conjugate (source and dilution)
GFAP	Thermofisher 1:1000	Biotinylated Goat anti-Chicken IgY	Jackson Immunoresearch, USA; 1:200	Streptavidin TRITC (Jackson Immunoresearch) 1:250
NU-1	William Klein (North Western University, IL) 1:1000	Biotinylated Goat anti-Rabbit IgG	Jackson Immunoresearch, USA; 1:200	Streptavidin TRITC (Jackson Immunoresearch) 1:250
mOC64	Abcam 1:500	Biotinylated Goat anti-Rabbit IgG	(Thermofisher), USA; 1:200	Streptavidin TRITC (Jackson Immunoresearch) 1:250
Ferritin	Sigma 1:500	Biotinylated Goat anti-Rabbit IgG	(Thermofisher), USA; 1:200	Streptavidin TRITC (Jackson Immunoresearch) 1:250

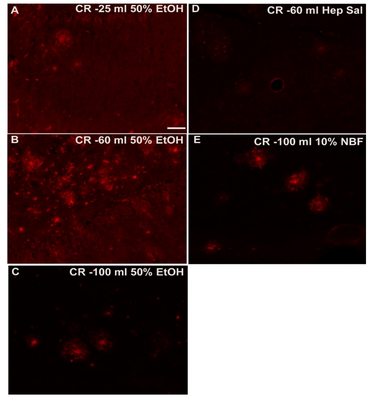

Figure 1. Figure 1. Images showcasing the different results from the five tested CR formulation variants.

Results and Discussion

We compared the staining results of five different CR formulations. The results from each formulation are described below:

(1) 0.5% CR in 25 ml of 50% EtOH perfused at a rate of 10 ml/min for a total of 2.5 minutes. We initially assessed the efficacy of CR perfusion using a formulation of 0.5% CR dissolved in 25 ml of 50% EtOH. In this formulation, CR dissolved easily, and there were no issues with the perfusion. The staining results exhibited some plaques, but a small amount of nonspecific vascular staining and some background staining and some degree of autofluorescence.

(2) 0.5% CR in 60 ml of 50% EtOH perfused at a rate of 20 ml/min for a total of 3 minutes. Next, we assessed 0.5% CR dissolved in 60 ml of 50% EtOH. In this case there were much more visible plaques, but more non-specific staining in the form of lipofuscin molecules.

(3) 0.5% CR in 100 ml of 50% EtOH perfused at a rate of 30 ml/min for a total of 3.3 minutes. We also assessed the staining efficacy of 0.5% CR in 100 ml of 50% EtOH . This formulation resulted in bright plaque staining with little to no non-specific staining or background staining.

(4) 0.5% CR in 60 ml of heparinized saline perfused at a rate of 20 ml/min for a total of 3 minutes.
Generally, our perfusion method utilizes heparinized saline (hep sal) followed by 10% neutral buffer formalin (NBF). Therefore, we wanted to determine the staining results if CR was dissolved these two solutions. First, we examined a formulation that consisted of 0.5% CR in 60 ml of heparinized saline. This formulation resulted in more of a suspension, rather than solution, but it did perfuse properly. Unfortunately, this formulation resulted in only very faint plaque staining . This formulation had the lowest staining intensity compared to the other four formulations.

(5) 0.5% CR in 100 ml of 10% neutral buffer formalin perfused at a rate of 30 ml/min for a total of 3.3 minutes. For method 5, 50 µl of EtOH was added to help with solubilizing the solution. Lastly, we assessed the staining intensity of 0.5% CR in 100 ml of 10% NBF . This formulation resulted in very bright plaque staining with little to no background staining. In order to determine possible reasoning underlying our worst result, variant (4) with our best result, variant (5), we examined the pH of these two formulations. Variant (4) had a pH of 8.87 and variant (5) had a pH of 7.39. Surprisingly, the second-best variant, variant (3) had a pH 9.0. Thus, for all five of our methods assessed, the 0.5% CR in 100 ml of 50% EtOH and 0.5% CR in 100 ml of 10% NBF exhibited the clearest results. Thus, we utilized tissue stained with those two methods for our subsequent experiments assessing compatibility with multiple-labeling procedures.

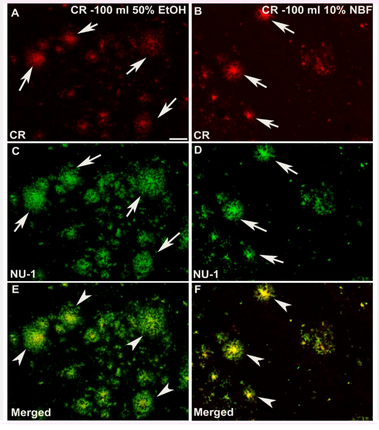

Figure 2. Images showcasing compatibility of CR Perfusion with mOC-64 and NU-1 staining, respectively. Arrows point to positive-labeled structures. Arrowheads point to areas of colocalization.

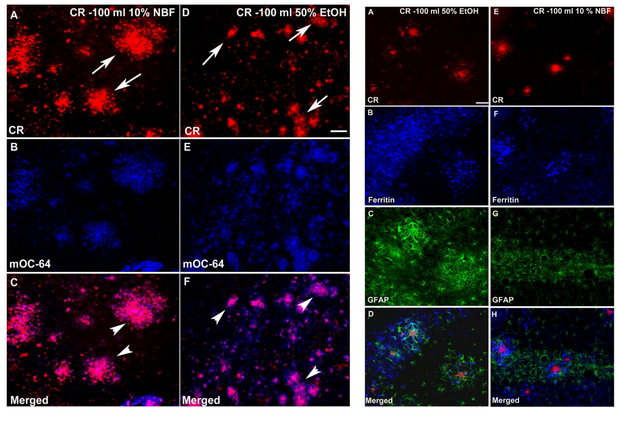

Figure 3 & 4. Images showcasing CR perfusion compatibility with multiple labeling procedures. Figure 4 shows an association between Congo red, Ferritin, and GFAP, as well as merged images.

Conclusion

1. **Congo Red perfusion labels parenchymal and vascular plaques throughout the brain**
2. **Congo Red perfusion is compatible with multiple-labeling procedures**
3. **Congo Red has near absolute colocalization with NU-1 which detects beta-amyloid oligomers**
4. **CR solutions that are alcohol- or formalin-based result in stable and intense beta-amyloid labeling**

Disclaimer

"The contents of this poster do not reflect any position of the U.S. Government or FDA."

FUNDING :This study was supported by NCTR protocol E0763101