

# Modeling transient retinal ischemia in mouse by ligation of pterygopalatine artery

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## KEY WORDS

Ischemia-reperfusion  
Mouse model  
Pterygopalatine artery  
External carotid artery

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## ABSTRACT

**Background:** Retinal ischemia is a major cause of visual impairment and blindness worldwide. The available therapeutic strategies have limited potential.

**Purpose:** In order to understand the pathophysiology and validating therapies for retinal ischemia, establishment of reproducible animal models is necessary.

**Methods:** In the model discussed in this article, the pterygopalatine artery (PPA) is ligated along with the external carotid artery for 3.5 hours and thereafter allowed to reperfuse. Because PPA supplies the blood to the ophthalmic artery, the ligation of this artery causes retinal ischemia.

**Results:** This article describes the validation of retinal ischemia-reperfusion model in mouse through PPA ligation and its validation through fluorescein fundus angiography (FFA) and immunofluorescence staining for glial fibrillary acidic protein (GFAP), a glial injury marker.

**Conclusions:** In conclusion this article describes the creation of mouse model of retinal ischemia-reperfusion injury which can be reproduced in a shorter time duration resulting in reduced mortality.

doi : 10.5214/ans.0972.7531.220406



## Introduction

Retinal ischemia is a major cause of blindness world-wide and is associated with various disorders such as diabetic retinopathy, glaucoma, stroke, optic neuropathy and other retinopathies.<sup>1</sup> It is caused by obstruction in blood supply to retina decreasing the oxygen and glucose supply. If

the blood supply is not restored, the damage becomes permanent leading to cell death and consequent vision loss. The damage caused to the retina can be due to various pathways which result in energy failure, excitotoxicity, ion channels imbalance, free-radical generation.<sup>2</sup> Reperfusion i.e. the restoration of blood flow to the retina leads to more damage to the retina than the ischemia itself. The re-establishment of blood supply to the ischemic retina increases the infiltration of macrophages and also leads to the generation of free-radicals causing more damage.<sup>3</sup>

Small animals, such as rats and mice are attractive models because they have vascular and phylogenetic similarities to humans. Besides, the availability, reproducibility and the relevance to humans are other factors that are important for use of mice for pre-clinical investigations.

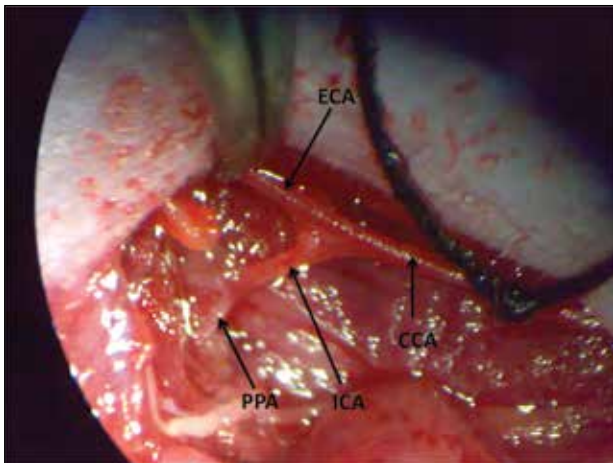
Many different approaches have been used in establishment of animal models of retinal ischemia. One approach is through the elevation of intraocular pressure (IOP).<sup>4</sup> In this method the IOP is increased above the systolic pressure. Selles-Navarro et al. quantified the retinal ganglion cells (RGC) survival at different time points in the high IOP model of retinal ischemia.<sup>5</sup> Photocoagulation of blood vessels is another means to cause retinal ischemia.<sup>6</sup> Another model for transient retinal ischemia involves temporary ligation of ophthalmic vessels. Lafuente et al. examined the RGC loss in this model of retinal ischemia by ligating the vessels for varying intervals of time and concluded

that the degree of RGC loss depends on the severity of injury caused.<sup>7,8</sup> In the same model, another study showed a significant decrease in the ERG wave amplitude along with the histological changes represented as decrease in inner retinal layer thickness.<sup>9</sup> Another approach includes middle cerebral artery occlusion (MCAO)<sup>10</sup> and chronic bilateral common carotid artery occlusion (BCCAO).<sup>11</sup> Some of these vascular models are based on the obstruction of blood supply to the ophthalmic artery which also receives the blood supply from the pterygopalatine artery (PPA) and the external carotid artery (ECA) in mice. The ligation of PPA along with the external carotid artery thus reduces the blood supply to the retina.<sup>12-14</sup> This information has been successfully exploited to establish mouse model of retinal ischemia.

Thus, this study describes the methodology for creation of mouse model of transient retinal ischemia and its validation through fluorescein fundus angiography and immunofluorescence analysis for glial fibrillary acidic protein (GFAP).

## Methods

The experiments were performed on 6 to 8 weeks old C57BL/6J male mice. The mice were anaesthetized using xylazine (50mg/ml) and ketamine (50mg/ml). The right common carotid artery and its branches were exposed after a midline incision (Figure 1). The external carotid artery (ECA) and the pterygopalatine artery (PPA), a branch of internal carotid artery (ICA) were ligated using suture (size 7-0; Ethicon, New Jersey, USA). After 3.5 hours of ligation, the blood supply was restored to allow reperfusion (Supplementary video S1). The changes in the cerebral blood flow were measured using Laser-doppler blood flow-meter (Moor Instruments, UK) by placing the probe in the cortical region on the skull on the ipsilateral side of the brain. The ligation was validated using fluorescein fundus angiography, where sodium fluorescein was injected intravenously after 45 minutes of ligation of PPA and fundus images of right and left eye were



**Fig. 1: Screen-shot for the mouse arteries.** The figure shows the right common carotid artery and its branches, where CCA- common carotid artery; ECA – external carotid artery; ICA – internal carotid artery; PPA – pterygopalatine artery.

captured using Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany).

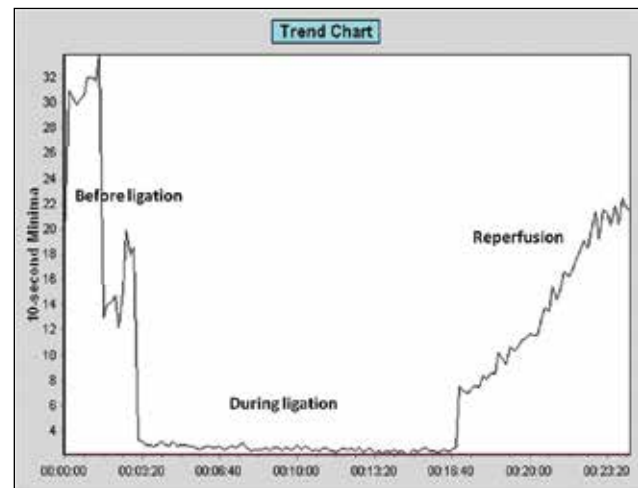
After 5 days of reperfusion, the mice were sacrificed and the eyes were enucleated. The cryosections were cut and placed on slides. The control and injured sections were blocked in the serum of animal species in which secondary antibody was raised and incubated with primary antibody for glial fibrillary acidic protein (GFAP) overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibody for 1 hour and counterstained with the nuclear stain, DAPI (1:1000, Life Technologies, USA). The sections were mounted with FluorSave reagent (Calbiochem, USA). The slides were observed under an upright fluorescent microscope (BX51, Olympus Corporation, Japan). The primary antibody used for immunofluorescence was GFAP goat polyclonal IgG antibody (1:100, Santa Cruz Biotechnology Inc., USA) and the secondary antibody was Cy3 conjugated donkey anti-goat antibody (1:200, Jackson ImmunoResearch Laboratories Inc., USA). The images were adjusted for contrast using Adobe Photoshop software.

All procedures were carried out following the GLP norms and according to the Standard Operating Procedures (SOP) established in the research facility. The experiments were approved by the Institutional Animal Ethical Committee (53/IAEC/273).

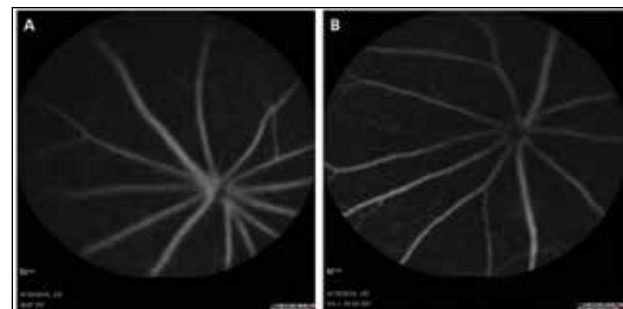
### Results

In this study, the changes in blood flow to the brain were examined using laser doppler ( $n = 3$ ). The laser doppler trend showed a decrease in blood flow during the ligation and was restored after the reperfusion of blood supply (Figure 2). The injury was further validated through fluorescein fundus angiography (FFA), where the fundus of the mouse after ligation was evaluated for fluorescein circulation ( $n = 4$ ). The fundus photographs after the ligation of the artery showed thinning of the vessels as compared to the contralateral eye (Figure 3).

The mice were sacrificed after 5 days of reperfusion and the validation of retinal ischemia model was done through histological and immunohistochemical analysis of retinal cryo-sections for injury markers such as glial fibrillary acidic protein (GFAP;  $n = 3$ ).



**Fig. 2: Blood flow measurement using Laser Doppler.** The trend chart from laser doppler blood-flow meter demonstrates the changes in the cerebral blood flow before ligation, during the ECA and PPA ligation and after the ligation is removed for reperfusion.



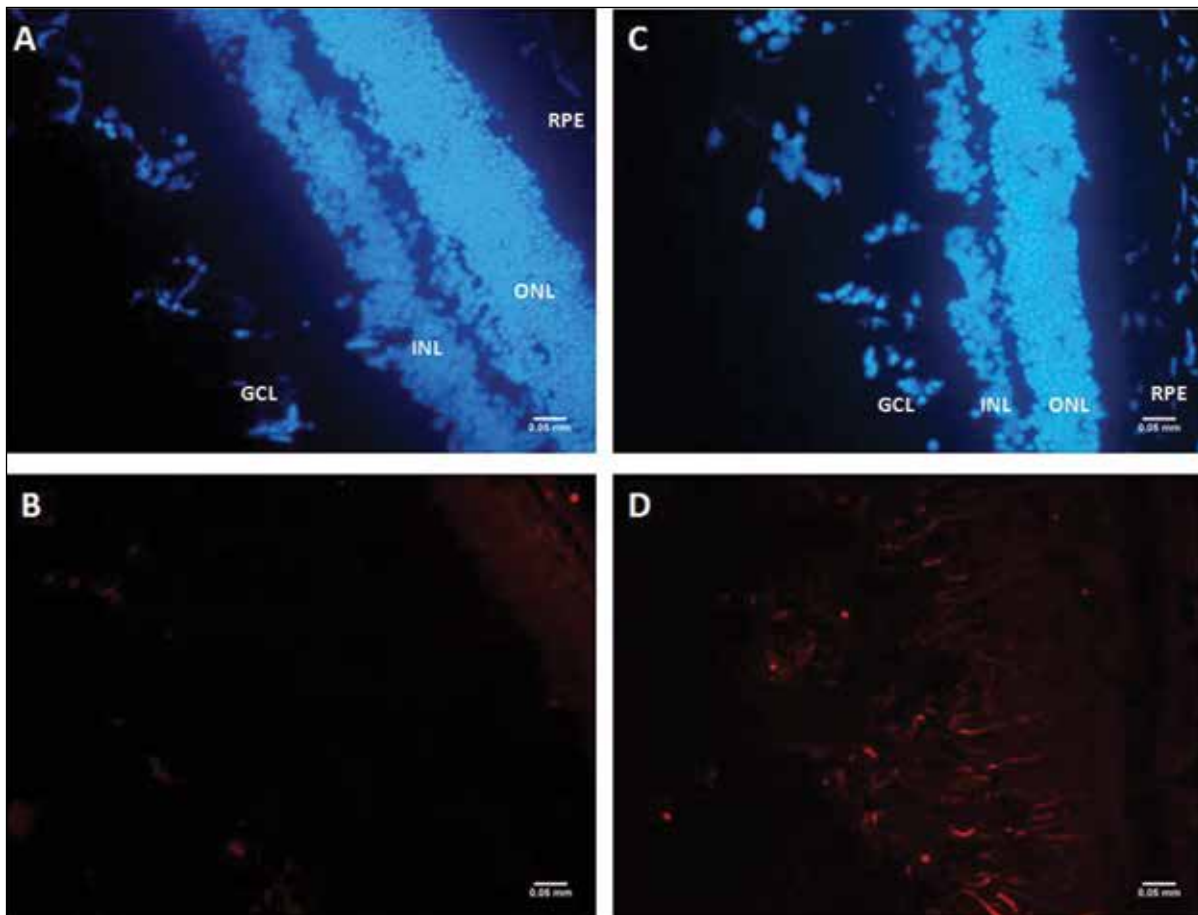
**Fig. 3: Fluorescein fundus angiography (FFA) of mouse eye.** The figure shows the fundus photographs of mouse eye after PPA ligation. A. Left eye is without PPA ligation; B. Right eye is after PPA ligation – which shows thinning of the vessels.

In this model of retinal ischemia, 3.5 hrs of ligation and 5 days of reperfusion was shown to have an increased expression of GFAP (Figure 4).

### Discussion

Retinal ischemia can be validated through histological and immunohistochemical analysis of retinal cryosections, quantitation of mRNA expression of injury markers in retina homogenate, blood flow measurements using laser doppler blood flow meter, electroretinography for electrophysiological changes and fluorescein fundus angiography.

The glial fibrillary acidic protein (GFAP) being a glial injury marker is known to be upregulated in response to neuronal injury and/or photoreceptor degeneration.<sup>15–17</sup> Osborne et al. have shown that the decrease in blood supply to retina caused by clamping of both carotid arteries led to increased GFAP expression which could be used as an index of retinal degeneration.<sup>18</sup> GFAP over expression has been reported in rat model of chronic BCCAO<sup>19</sup> as well as in the optic nerve crush model.<sup>20</sup> Eisenfeld et al. reported elevated GFAP expression in RCS rats with genetic retinal degeneration.<sup>21</sup>



**Fig. 4: Immunofluorescence of GFAP expression after PPA ligation.** Immunohistochemical staining for glial fibrillary acidic protein (GFAP) in cryosections for control (A-B) and experimental mice (C-D); GFAP is stained with Cy3; blue is DAPI which stains the nuclei (Scale bar – 50 microns).

The mouse model of retinal ischemia reported in this article involves the ligation of both the external carotid artery and the pterygopalatine artery for the stipulated time-period. This leads to retinal damage by transient ischemia-reperfusion injury. This model has advantages over the other models that are being used to study retinal ischemia. The model established by elevation of intraocular pressure causes damage to retina through both ischemia and high pressure<sup>22,23</sup> unlike the PPA ligation model which is more specific and well defined. The photocoagulation model, on the contrary, cannot be used to study transient ischemia as the damage caused is permanent. The middle cerebral artery occlusion model leads to both cerebral and retinal ischemia thus enhancing the mortality rate in the experimental animals.<sup>24</sup>

This model can be created in short period of time and has low mortality rate. The few confounders that need to be taken care of include the body temperature, infection, feed and housing conditions. The model can also be modified for the degree of retinal injury caused by changing the duration of ligation as well as reperfusion and can be further used to test new drugs and other therapeutic strategies for retinal ischemia.

#### Authorship Contributions

**Gillispie Minhas:** Performed the surgery **Ryuichi Morishita,** **Munehisa Shimamura:** Provided training, **Reema Bansal:**

Supervised the FFA, **Akshay Anand:** Designed, supervised and provided resources for the study.

#### Acknowledgements

The authors acknowledge the Department of Science and Technology (F.No. SR/SO/HS-0001/2010), Council of Scientific and Industrial Research, India and Disease Models and Mechanisms Fellowship, London UK.

This article complies with International Committee of Medical Journal editor's uniform requirements for manuscript.

Conflict of interest: None; Funding: None.

Competing Interests: The authors declare no competing interests.

Received Date : 22 February 2015; Revised Date : 14 March 2015;

Accepted Date : 23 March 2015

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