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# Protective effect of vitreous against hemoglobin neurotoxicity

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ABSTRACT

Hemorrhage into the brain parenchyma or subarachnoid space is associated with edema and vascular injury that is likely mediated at least in part by the toxicity of hemoglobin. In contrast, extravascular blood appears to be less neurotoxic when localized to the retina or adjacent vitreous, the gel filling the posterior segment of the eye. In this study, the hypothesis that vitreous protects neurons from hemoglobin toxicity was investigated in a primary cortical cell culture model. Consistent with prior observations, hemoglobin exposure for 24 h resulted in death of most neurons without injury to co-cultured glia. Neuronal loss was reduced in a concentration-dependent fashion by bovine vitreous, with complete protection produced by 3% vitreous solutions. This effect was associated with a reduction in malondialdehyde but an increase in cell iron. At low vitreous concentrations, its ascorbate content was sufficient to account for most neuroprotection, as equivalent concentrations of ascorbate alone had a similar effect. However, other vitreous antioxidants provided significant protection when applied at concentrations present in undiluted vitreous, and prevented all neuronal loss when combined in the absence of ascorbate. These results indicate that vitreous is an antioxidant cocktail that robustly protects neurons from hemoglobin toxicity, and may contribute to the relative resistance of retinal neurons to hemorrhagic injury.



## 1. Introduction

Hemoglobin (Hb) is a pro-oxidant protein that is released into CNS tissue in millimolar concentrations after spontaneous or traumatic hemorrhage. Its location within erythrocytes provides an effective barrier that prevents its toxicity within the circulation, and also in the initial hours after parenchymal or subarachnoid hemorrhage. However, subsequent erythrophagocytosis by microglia and infiltrating macrophages is apparently insufficient to prevent significant local Hb release and breakdown. At one week after experimental subarachnoid hemorrhage, heme concentrations within the hematoma are two orders of magnitude above those required to kill cultured neurons [1], the cell population most vulnerable to Hb and iron [2,3]. In vivo, parenchymal injection of autologous blood or Hb produces a delayed iron-dependent injury that is attenuated in rodent and pig models by the ferric chelator deferoxamine [4–7].

In contrast to its toxicity in the brain parenchyma and subarachnoid space, the deleterious effects of extravascular blood appear to be mitigated in the eye. Retinal neurons and photoreceptors sustain relatively little injury after hemorrhage localized to the retina or extending into the vitreous [8], the hyaluronan-based gel in the posterior segment of the eye. Accordingly, management is primarily conservative, limited to postural changes to promote erythrocyte settling or observation alone, and usually results in a satisfactory outcome [9,10]. While this phenomenon may merely indicate that retinal cells are selectively resistant to heme or iron-mediated injury, two observations suggest otherwise. First, photoreceptor degeneration is observed when hemorrhage is localized to the subretinal space rather than the retina or vitreous, and can be reduced by deferoxamine [11–13]. Second, the vulnerability of cultured retinal neurons to iron resembles that of other central neurons [3,14,15].

An alternative hypothesis is that vitreous is inherently protective against Hb, and reduces the vulnerability of adjacent cells to its oxidative toxicity. If that is so, then elucidation of its protective mechanisms may have implications beyond ocular hemorrhage,

*Abbreviations:* Hb, hemoglobin; LDH, lactate dehydrogenase; PI, propidium iodide.

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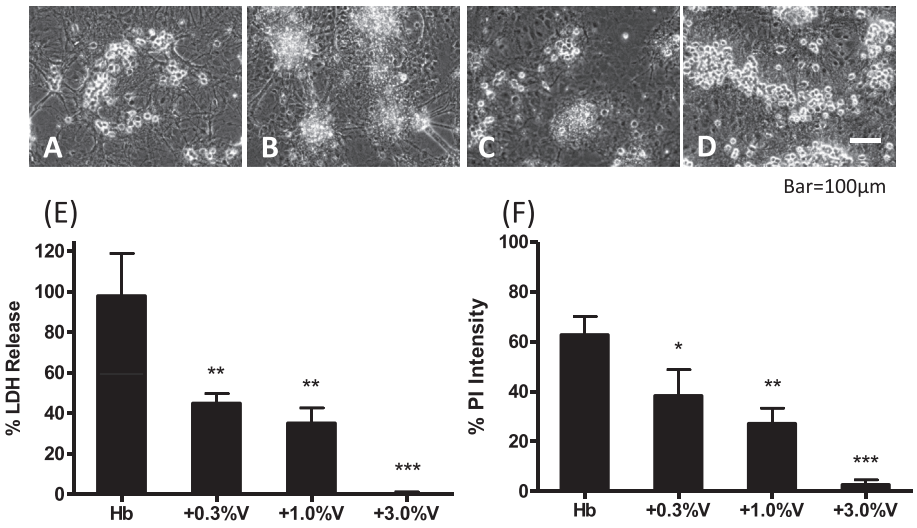


**Fig. 1.** Vitreous protects cortical neurons against hemoglobin (Hb) toxicity. A-D) Phase contrast photomicrographs of cultures after 24 h exposure to: A) sham media exchange; phase-bright neuronal cell bodies overlie a confluent glial monolayer; B) Hb 10 μM; most neuronal cell bodies have degenerated; C,D) Hb 10 μM plus 0.3% and 3% bovine vitreous dilutions, respectively; concentration-dependent protection is apparent. E, F) Bars represent mean percentage cell death (±SEM), as measured by LDH release (E) and propidium iodide fluorescence assays (PI, F), in cultures treated for 24 h with Hb 10 μM alone or with indicated bovine vitreous (V) dilutions. The low mean LDH or background fluorescence values in sham cultures subjected to medium exchange only were subtracted from each value to determine the signal specific for Hb neurotoxicity. *P < 0.05, **P < 0.01, ***P < 0.001 v. Hb alone conditions, Bonferroni multiple comparisons test, n = 8–9 cultures/condition).

and may provide information relevant to the design of safe and effective therapies for hemorrhagic stroke and trauma. As an initial step towards this end, we investigated the effect of bovine vitreous in a characterized model of Hb neurotoxicity.

## 2. Materials and methods

### 2.1. Materials

Bovine vitreous was purchased from InVision Bioresources, Seattle, Washington, USA. It was frozen after harvesting and shipped on dry ice. For use in experiments, it was quickly thawed, homogenized while ice-cold, and sterile-filtered. Aliquots were then stored at −80 °C until used.

Human Hb A was obtained as a gift from Hemosol, Inc, Etobicoke, Ontario, Canada.

Apotransferrin was purchased from Millipore-Sigma, Burlington, MA, USA and apoferritin was purchased from Sigma-Aldrich, St Louis, MO, USA.

Hyaluronic acid was purchased from R&D Systems, Minneapolis, MN, USA.

Culture media (MEM and DMEM) were purchased from Gibco-Thermo Fisher Scientific, Gaithersburg, MD, USA; serum was purchased from GE Healthcare Hyclone, Logan, UT, USA.

Other experimental reagents were purchased from Sigma-Aldrich unless otherwise indicated.

### 2.2. Primary cell cultures

Mouse breeding, rearing and culture preparation followed protocols approved by the Thomas Jefferson University Institutional Animal Care and Use Committee, and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Cultures containing neurons and glia (>90% GFAP + astrocytes) were prepared from the pooled cortices of fetal C57BL/6 × 129/Sv mice that were collected at gestational age 14–16 days, as previously described [16]. After trypsin digestion, tissue was dissociated to a cell suspension by trituration and diluted in medium containing 5% fetal bovine serum, 5% equine serum, 2 mM

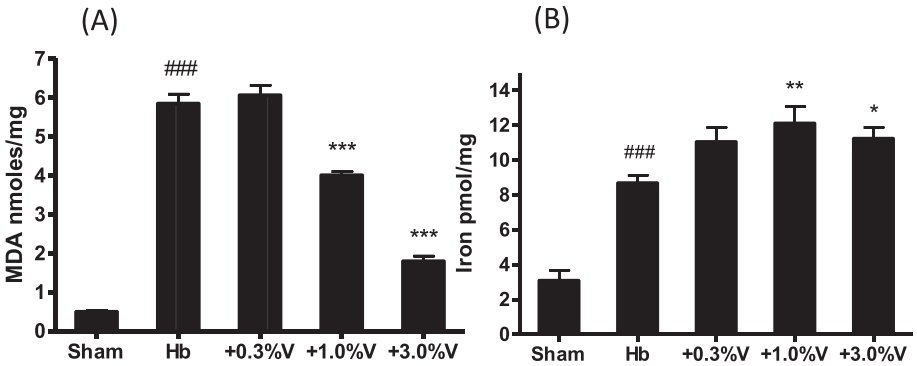


**Fig. 2.** Vitreous reduces cell malondialdehyde (MDA) but increases iron after hemoglobin exposure. Mean malondialdehyde (A, 10 cultures/condition) and iron (B, 8–10 cultures/condition) after treatment for 24 h with Hb 10 μM alone or with indicated bovine vitreous dilutions. *P < 0.05, **P < 0.01, ***P < 0.001 v. Hb alone conditions, ###P < 0.001 v. sham, Bonferroni multiple comparisons test.

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