Effect of Melanin on Traumatic Hyphema in Rabbits

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Objective: To investigate the role of melanin in influencing the clearance of traumatic hyphema and in the incidence of rebleeds following the hyphemas.

Methods: Hyphemas were induced in 30 eyes of New Zealand white albino rabbits using an Nd:YAG laser. A total of 3.75 mg of synthetic melanin suspended in 0.1 mL of balanced salt solution was introduced into the anterior chambers of 16 animals. A total of 0.1 mL of balanced salt solution was injected into 14 control eyes. Hyphema levels were measured by a masked observer (V.D.B.) daily for 15 days. Pairs of animals were sacrificed at 1, 3, 5, 10, and 15 days and the eyes studied histologically.

Results: Hyphemas were consistently produced in all eyes with mean ± SD levels of 1.44 ± 0.22 mm and 1.57 ± 0.24 mm in the melanin-treated and control eyes, respectively. The clearance of hyphemas in the melanin-treated eyes was significantly prolonged throughout the study (P<.001). The rate of rebleed in the melanin-treated group was 18.8% and in the control group was 7.1% (P<.01). Histologically, both groups showed variable degrees of blood in the anterior chambers and trabecular meshwork. In addition, the melanin-treated eyes showed free melanin, melanin-laden macrophages, and an inflammatory response in the anterior chamber and trabecular meshwork that was greater than that in the control eyes. Melanin-treated eyes with rebleeds showed organized hemorrhage with neovascularization.

Conclusions: The presence of melanin results in a significantly prolonged course of hyphemas and may influence the rate of rebleeds. Occlusion of the trabecular meshwork with melanin-laden macrophages and inflammation may be the mechanisms responsible for these effects.

Clinical Relevance: The release of melanin into the anterior chamber during ocular trauma may be partly responsible for the susceptibility of darker-pigmented individuals to more serious complications following a traumatic hyphema.


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MATERIALS AND METHODS

All procedures involving animals were performed according to the institutional guidelines established by the University of Illinois at Chicago and followed the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Vision Research. The study was conducted in 2 phases, an in vitro and an in vivo study phase. Data are presented as mean ± SD.

IN VITRO STUDIES

Range of Whole Blood Clotting Time in Rabbits

The range of whole blood clotting time was determined according to methods described previously. Briefly, 5 New Zealand white albino rabbits were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride (40 mg/kg) and acepromazine maleate (0.5 mg/kg). Two milliliters of blood were drawn from the aural vein of each animal using a 19-gauge needle attached to a 5-mL disposable syringe. Two milliliters of blood were placed in each of 2 glass test tubes immersed in a water bath at 37°C. A stopwatch was started, and the test tubes were inspected every 15 seconds by gentle tilting until they could be inverted with no blood flowing down the side of the tube. The time it took for this to occur was noted.

Effect of Melanin on Clotting Time

Rabbit blood was obtained by repeating the procedure described herein. Synthetic melanin prepared by oxidation of tyrosine with hydrogen peroxide was used in the study (Sigma-Aldrich Corp, St Louis, Mo). The melanin was sterile and non-pyrogenic. Varying quantities of melanin ranging from 25 to 100 mg and suspended in 2 mL of balanced salt solution (BSS) were added to 2 mL of the blood samples. Clotting times were noted, and 2 samples were tested at each time point.

IN VIVO STUDIES

Thirty New Zealand white albino rabbits (weighing 2-3 kg each) were used in the study. They were anesthetized by intramuscular injections of a mixture of ketamine hydrochloride (40 mg/kg) and acepromazine maleate (0.5 mg/kg). Topical 0.5% proparacaine hydrochloride (40 mg/kg) and acepromazine maleate (0.5 mg/kg). Two milliliters of blood were drawn from the aural vein of each animal using a 19-gauge needle attached to a 5-mL disposable syringe. Two milliliters of blood were placed in each of 2 glass test tubes immersed in a water bath at 37°C. A stopwatch was started, and the test tubes were inspected every 15 seconds by gentle tilting until they could be inverted with no blood flowing down the side of the tube. The time it took for this to occur was noted.

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RESULTS

IN VITRO STUDIES

Mean clotting time of whole blood was 6.39 ± 1.77 minutes. Following the addition of the melanin suspension, clotting time decreased in a dose-dependent fashion until 100 mg of melanin suspension and 2 mL of blood were added (Figure 1). Mean clotting time of blood treated with melanin was 3.46 ± 1.78 minutes, with a range of 1.23 to 6.29 minutes. Linear regression analysis revealed the slope of the graph to be 0.067 minutes per milligram of melanin per 4 mL of suspension ($R = 0.999$). A 95% confidence level was obtained of this being different from a slope of zero.

IN VIVO STUDIES

Hyphemas were consistently produced in all laser-treated eyes (Figure 2). Mean initial hyphema levels were 1.44 ± 0.22 mm and 1.57 ± 0.24 mm in the melanin-treated eyes. Following the production of the hyphema, a paracentesis of 0.1 mL was performed. A total of 3.75 mg of melanin that was suspended in 0.1 mL of BSS was injected into the anterior chambers of 15 animals using a 30-gauge needle attached to a tuberculin syringe. This amount was determined by the in vitro data plot using the weight of melanin that produced the maximal influence on clotting time and taking into consideration the amount of melanin that could be practically suspended in 0.1 mL of saline. Specifically, 75 mg of melanin that was suspended in 2 mL of BSS and 2 mL of rabbit blood (ie, 18.75 mg/mL) was chosen. This was adjusted to the volume of aqueous in the anterior chamber (200 µL) of the rabbit eye and the volume of BSS it could be suspended in—yielding a final weight of 3.75 mg. As a control, 0.1 mL of BSS was injected into the anterior chambers of the remaining 15 animals.

A masked observer (V.D.B.), using a calibrated steel ruler, measured the level of hyphema daily. A rebleed was defined as an increase in hyphema level of more than 2 mm in 24 hours. Pairs of animals, one experimental and one control, were sacrificed using an overdose of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Ill) at 1, 3, 5, 10, and 15 days. Their eyes were enucleated and fixed overnight in 4% buffered formaldehyde. The globes were opened vertically and the pupillary–optic nerve sections were submitted for routine processing and paraffin embedding. Deparaffinized sections, 5-µm thick, were then obtained for light microscopic evaluation.

STATISTICAL ANALYSIS

In Vitro Studies

The slope of the graph plotting melanin concentration against clotting time was calculated. Linear regression was performed, and the slope was statistically compared with a slope of zero.

In Vivo Studies

The levels of hyphema and clearance were statistically compared using the analysis of variance method. The rates of rebleed between the melanin-treated and control groups were compared using the $\chi^2$ test.
and control eyes, respectively. There was no statistical difference in initial mean hyphema level between the melanin-treated and control groups. Clearance of hyphema was significantly prolonged in the melanin-treated eyes \((P<.001)\) throughout the study (Figure 3). Mean clearance periods were \(7.17 \pm 0.69\) days in the melanin-treated group and \(3.18 \pm 0.64\) days in the control group. The rate of rebleed was significantly higher in the melanin-treated group \((19\%, n = 16)\) than in the control group \((7\%, n = 14)\) \((P<.01)\).

At 1 and 3 days after treatment, the saline-treated eyes showed fibrin mixed with aggregates of red blood cells (Figure 4, left). In the melanin-treated eyes, we observed free melanin and melanin-laden macrophages mixed with fibrin in the anterior chamber and trabecular meshwork (Figure 4, right). Five days after treatment, scant red blood cells were noted in the anterior chamber angle of the control eyes. In the melanin-treated eyes, free melanin, melanin-laden macrophages, red blood cells, and fibrin were observed in the anterior chamber and angle. At 10 days after treatment, most melanin-treated eyes showed melanin-laden macrophages in the anterior chamber angle, trabecular meshwork, and Schlemm canal. No red blood cells were noted in these eyes, but in some, organized hemorrhage was noted on the surface of the iris with neovascularization and mild inflammation (Figure 5). Fifteen days after treatment, the red blood cells were largely cleared from the anterior chamber in both groups. Melanin-laden macrophages were seen in the melanin-treated eyes on the iris surface. In all melanin-treated eyes, a greater number of lymphocytes was seen mixed with melanin, fibrin, and macrophages than with the control eyes.

**COMMENT**

The major complications of traumatic hyphema include rebleeding, glaucoma, and corneal blood staining. Rebleeding is a major concern because it is associated with a poor prognosis of the eventual visual outcome.\(^4,17,20\) It has been our clinical impression that the course of hyphema is prolonged and the incidence of rebleeds is higher in the black population. Several studies\(^6,9,14,17\) have also reported that darker-pigmented individuals appear to be more susceptible to developing rebleeds, and hyphemas in this population may be associated with more complications and a worse visual outcome. We hypothesized that such susceptibility may be related to the release of melanin into the anterior chamber during ocular trauma. Our results are compatible with this hypothesis.

In the first part of this study, we examined the effect of melanin on the clotting of whole blood obtained from albino rabbits. We demonstrated that melanin significantly decreased the mean clotting time of whole blood in a dose-dependent fashion. The acceleration of clot formation by melanin may partly explain our clinical observation of the prolonged clearance of hyphemas in darker-pigmented eyes. While the exact mechanism responsible for this effect is not fully known, the interfer-
ence of clot formation may be due to an activation of the clotting cascade following contact of melanin with clot-activating factors. Ocular melanin granules are polymeric units of melanin shown to possess the capacity to bind various drugs and possibly other physiologic substances. The unique binding properties of melanin may allow it to bind factors released from local blood vessels and facilitate the production and retention of a clot in the anterior chamber.

The second part of our study examined the effect of melanin on the clearance of hyphemas and the incidence of rebleed following the hyphemas in the eyes of albino rabbits. We showed that clearance of the hyphemas was significantly prolonged in melanin-treated eyes throughout the study period. One possible mechanism by which the clearance of blood was prolonged may be partly explained by our in vitro finding of the effects of melanin on clotting time. It is possible that free melanin and/or macrophages may have accelerated blood clotting in the anterior chamber that thereby led to prolonged retention of blood in the anterior chamber. This finding could not be qualitatively demonstrated by histopathologic review of the enucleated specimens since

Figure 3. Levels of hyphema in the melanin-treated and saline-treated eyes. The clearance of the hyphema was significantly prolonged in the melanin-treated group when compared with that of the saline-treated group (P < .01). The variations represented on the graph reflect the SEM.

Figure 4. Photomicrographs showing the anterior segment of the rabbit eyes 1 day after laser treatment. Left, In the saline-treated controls, aggregates of red blood cells mixed with fibrin over the anterior surface of the iris and angle were noted (hematoxylin-eosin, original magnification ×40). Inset, Higher magnification showing red blood cells in the angle recess and trabecular meshwork (hematoxylin-eosin, original magnification ×100). Right, In the melanin-treated eye, large aggregates of red blood cells surrounded by fibrin, free melanin, and melanin-laden macrophages were seen (hematoxylin-eosin, original magnification ×40). Inset, Anterior chamber angle at higher magnification showing melanin-laden macrophages (hematoxylin-eosin, original magnification ×100).

Figure 5. Photomicrographs of the anterior segment 10 days after treatment. Left, In the saline-treated eyes, the red blood cells have largely cleared from the anterior chamber (hematoxylin-eosin, original magnification ×40). Right, In the melanin-treated eyes, organized hemorrhage mixed with free red blood cells is noted over the anterior surface of the iris. Neovascularization is seen near the organized hemorrhage (hematoxylin-eosin, original magnification ×40). Inset, Higher magnification showing area of neovascularization (arrow) (hematoxylin-eosin, original magnification ×160).
both groups showed variable amounts of fibrin in the anterior chamber. By histopathologic review, the melanin-treated eyes enucleated in the early postlaser treatment period revealed aggregates of red blood cells surrounded by free melanin and melanin-laden macrophages in the anterior chamber and trabecular meshwork. A similar infiltration of macrophages was observed in melanomalytic and pigmented glaucomas. In these conditions, melanin granules that are liberated into the anterior chamber are then phagocytized by macrophages. Such melanin-laden macrophages then mechanically obstruct the anterior chamber angle and may be responsible for the impedence of outflow and the development of secondary open-angle glaucoma in these conditions. Mechanical obstruction of the outflow facility by melanin-laden macrophages may explain the prolonged clearance of the hyphema observed in our study. In agreement with this theory was the observation that, in the late posttreatment period, melanin-laden macrophages were noted in the anterior chamber and the trabecular meshwork of melanin-treated eyes. In contrast, the red blood cells had largely cleared from the anterior chamber and trabecular meshwork of saline-treated eyes.

In melanin-treated eyes, an inflammatory response was also noted in the anterior chamber and trabecular meshwork that was greater than in the control eyes. This may further contribute to a delayed clearance of the hyphema. Kaya and coworkers have shown that the presence of free melanin introduced into the anterior chamber could increase intraocular inflammation. Although the mechanism by which melanin augments inflammation is not known, they suggested that the binding of melanin to serum components may contribute to its proinflammatory effect.

We noted a greater incidence of rebleeding in melanin-treated eyes. This suggests that melanin may play a partial role in causing this complication. However, our examination of these eyes did not reveal a definite histologic explanation of rebleeding. In some animals, organization of hemorrhage with neovascularization was noted that might partially explain the increased incidence of rebleeding in the rabbits. It is unlikely that this mechanism is responsible for the rebleeding observed in humans. As stated previously, melanin has unique binding properties for many molecules. One could speculate that a presence of melanin in the anterior chamber will modulate fibrinolytic activity that will in turn lead to early clot lysis and rebleeds. In actual trauma, there is also probably greater tissue disruption with necrosis of iris melanocytes and the iris pigment epithelium and release of various inflammatory mediators. The influence of such factors on rebleeds was not addressed in this study.

In summary, this experimental study suggests that melanin affects the clearance of red blood cells and the rate of rebleed in traumatic hyphema. Further studies need to be performed to define the exact mechanism involved in the process of rebleeds.

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