Srp Arh Celok Lek. 2013 Nov-Dec;141(11-12):732-737
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1312732V
UDC: 617.7-005.1-085.357.55

Natural Evolution of Experimental Vitreous Hemorrhage and Effects of Corticosteroids on Its Course

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SUMMARY

Introduction The course and prognosis of vitreous hemorrhage (VH) are difficult to predict in spite of so far published experimental and clinical studies.

Objective The goal of this study was to follow the course and evaluate prognosis of vitreous hemorrhage.

Methods The experiment was performed on 19 Chinchilla rabbits (*Oryctolagus cuniculus*), both males and females (38 eyes), weighing 2500-3000 g, which were treated with autologous blood (B) from marginal ear vein – the first group, and the second group was treated with both blood and corticosteroids (B+CS). The course of vitreous hemorrhage was examined.

Results Our results have shown that the cellular reaction to vitreous hemorrhage is different, compared to hemorrhage in other types of tissue, which is due to absence of an early polymorphonuclear cellular reaction. Number of cells within vitreous body is very low.

Conclusion Vitreous hemorrhage has unusual course followed by a small number of cells (mostly polymorphonuclear cells). This is most important fact why VH is cleared very slowly (about 6 to 9 weeks). CS has moderate influence on the acceleration of VH absorption.

Keywords: vitreous hemorrhage; natural course; corticosteroids; rabbits

INTRODUCTION

In our practice it is very difficult to predict final results when vitreous hemorrhage (VH) is present. This was the main accelerator to conduct this experiment and show the real influence of CS in the clearance rate of VH using Chinchilla rabbits. Hemorrhage within the vitreous body in all cases originates from the periphery, or from the vessels penetrating into the vitreous in certain pathological conditions, thus leading to the condition known as vitreous hemorrhage, or hemophthalmus.

Vitreous is a dilute meshwork of collagen fibrils interspersed with the extensive arrays of hyaluronan molecules. The collagen fibrils provide a scaffold-like structure that is inflated by the hydrophilic hyaluronan. If collagen is removed, the remaining hyaluronan forms a viscous solution. However, if hyaluronan is removed, the gel shrinks but is not destroyed. Electrostatic binding occurs between the negatively charged hyaluronan and the positively charged collagen in the vitreous [1].

Studies have shown that the chondroitin sulfate chains of type IX collagen bridge between the adjacent collagen fibrils in a ladder-like configuration spacing them apart [2]. Such spacing is necessary for vitreous transparency, because keeping vitreous collagen fibrils separated by at least one wavelength of incident light minimizes light scattering, allowing the unhindered transmission of light to the retina for photoreception [3].

The vitreous forms a diffusion coefficient that allows drug and hemorrhage to remain.

Various enzymes have been investigated to dissolve the vitreous hemorrhage or modify the structural characteristics of the vitreous to allow diffusion of blood out of the visual axis.

Vitreous collagens are organized into fibrils with types V/XI residing in the core, type II collagen surrounding the core, and type IX collagen on the surface of the fibril. The fibrils are 7 to 28 nm in diameter, but their length in situ is unknown. Studies have shown that the vitreous contains collagen type II, a hybrid of types V/XI, and type IX collagen in a molar ratio of 75:10:15, respectively [4].

The primary role of corticosteroids is minimization of the inflammatory response. Their action is, according to modern knowledge, local, which means corticosteroids must be at the site of infection. There, corticosteroids lessen increased permeability of endothelial capillary cells, thus reducing the leakage of fluids and proteins transport at the site of lesion. The glucocorticoid receptor (GR) is a protein that mediates the actions of all pharmaceuticals. GRs regulate metabolism and are most well known for their role in inflammation [5]. It is at the very top of the inflammation pathway working in a multidirectional manner to subside inflammation. There are eight GR subtypes, and all are produced from a single messenger RNA [6, 7].

Injection of CS into the vitreous cavity stabilizes blood retinal barrier and helps clearing

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the vitreous hemorrhage. Dexamethasone in humans appears to have 4-month clinical duration, but in animals and *in vitro* it lasts to 6 months. There is a lack of basic understanding of the GR biochemistry in the eye, in terms of types of cells and their functions and, therefore, more research is required [5].

The assessment of hemolysis was based on its cardinal sign – vitreous opacification. Fibrinolysis was indirectly assessed by analyzing the size of the coagulum.

OBJECTIVE

Cellular response to vitreous B(blood), B+CS (blood + corticosteroid) was analyzed by counting and establishing an average cell number seen within 3 visual fields at characteristic spots, using large magnification (400×), evaluating the natural course of vitreous hemorrhage through coagulum disintegration and any modifications probably induced by CS.

METHODS

The experiment was carried out on 19 adult Chinchilla rabbits (*Oryctolagus cuniculus*) of both sexes, weighing 2500-3000 g. The animals were anesthetized by intramuscular injection of ketamine hydrochloride, using dose of 14-15 mg/kg. Both eyes were softened by withdrawal of 0.15 ml of liquid vitreous using 27-gauge needle and tuberculin syringe. By means of plastic silicone coated syringe, 0.2 ml of full autologous blood was collected from the marginal ear vein without using anticoagulants. The blood was immediately injected into the central part of the vitreous body, using standardized procedure of *via pars plana* approach to the upper temporal quadrant, 3 mm away from the limbus. The procedure was performed under ophthalmoscopic control and using 27-gauge needle. Eyeballs were perforated by sclerotomy, in all cases using 19 gauge needle and *pars plana* approach, 3 mm away from the limbus in the upper temporal quadrant.

The animals (eyes) were categorized into two basic groups: first group (right eyes) – full autologous blood was injected into the central vitreous body only; second group (left eyes) – full autologous blood was administered, plus 0.15 ml of intravitreous dexamethasone sodium phosphate (Dexason, ICN Galenika) or 0.3 mg through the same incision through sclera, 7 minutes and 7 days after injection of full blood, respectfully. The first group of eyes, in which blood was injected and the evolution of coagulum was analyzed in detail, was at the same time the control group to another group of animals (eyes), which suffered the same traumatic procedure of intravitreous injection of blood, and B+CS.

All experimental animals received blood injection in a manner described above, while group 2 was administered 0.15 ml of dexamethasone solution intravitreally, in the way that one subgroup received it after 5 minutes and another after 7 days.

An experimental group, intended for assessment of possible adverse effects of ocular trauma (or mini control group), consisted of 2 rabbits (4 eyes). In that group, an experimental wound was created on the right eye of the first rabbit, in *pars plana,* 3 mm away from the limbus, using 27-gauge needle. On the left eye, a wound was created using 19-gauge needle, 3 mm away from the limbus, with simultaneous intravitreous injection of 0.2 ml of physiological normal saline solution In the second rabbit, *pars plana* wound of the right eye was created using 27-gauge needle, with simultaneous aspiration of 0.15 ml of liquid vitreous substance. On the left eye, 3 mm away from the limbus, a wound was created using 19-gauge needle, with simultaneous injection of 0.2 ml of normal physiological solution.

A mini control group was evaluated using ophthalmoscopy and in certain periods of time the eyeballs were frozen in acetone after enucleation, and then anatomically and physiologically analyzed.

The interventions were completed by administration of 1% atropine solution and 1% chloramphenicol oil suspension into the conjunctival sac.

Based on literature [8, 9], time intervals after which it was found that the material was useful for investigation were: 5 minutes, 6 days, 2, 3, 4, 6, 9, 10, and 12 weeks after intravitreous injection of blood.

The animals were eutinized using ketamine overdose or by causing gas embolism.

Blood clearance from the vitreous body was assessed weekly, using an indirect ophthalmoscopy and making drawings of the observed image, until week 12 after intravitreous injection of blood. In addition, drawing of ophthalmoscopic findings was made 5 minutes after the intravitreous injection of blood.

The eyeballs were enucleated using standard technique.

Twenty-four eyeballs were immediately placed into 4% neutral buffered formaldehyde for fixation, while 9 eyeballs (rabbit 6 had only one eye) were frozen in PVC bags with acetone, by application of liquid nitrogen for 30 seconds.

The eyeballs fixed in formaldehyde were cut through the frontal plane, behind the lens, while the eyeballs frozen in acetone were cut vertically, through the sagittal plane, so the topographic characteristics of changes in vitreous body would remain preserved. For histological analysis, vitreous coagula together with the surrounding tissue, wedge-like slices of eyeball tissue with parts of ciliary body and adjacent tissue, parts of retina, as well as granulomas or vitreoretinal proliferative bands, if any, were sampled.

All slices were stained using haematoxylin & eosin (HE), periodic acid-Schiff (PAS) and van Gieson (VG) staining methods.

For data analysis, the following statistical methods were used: structural analysis, average, standard deviation, standard error of the average, confidence interval for p=0.95, Kaplan-Meier test, trend lines and trend errors, correlation coefficient (r), determination coefficient (r^2), and indetermination coefficient (1-r).

RESULTS

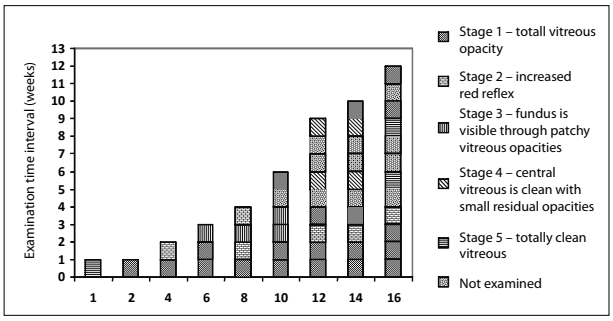
Two groups of eyes (rabbits) were examined: those treated with blood only (B), and those treated with blood and corticosteroids (B+CS). Ophthalmoscopic appearance of vitreous body was recorded using drawings in two groups of eyes.

The extent of vitreous blood elimination, which was assessed ophthalmoscopically, was arbitrarily segregated in five stages: stage 1 – total vitreous opacity; stage 2 – increased red reflex; stage 3 – fundus is visible through the patchy vitreous opacities; stage 4 – central vitreous is clean with the presence of small residual opacities; and stage 5 – totally clean vitreous.

The characteristics of elimination processes in two groups treated with B and B+CS were shown in Graphs 1 and 2. In rabbits treated with blood only, the changes of vitreous opacities started during week 4 after the injection. Almost all rabbits, which survived the experiment, reached stages 4 or 5 (considering it normal finding) after week 6, as shown in Graph 1. The course of the vitreous hemorrhage after injection of B+CS was shown in Graph 2. Vitreous clearance started in the third week, while all rabbits, which survived, reached stages 4 or 5 after the week 3.

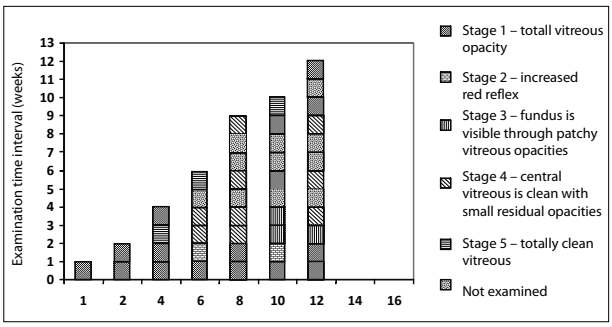
Table 1 shows that, when the injected substance was blood, the coagulum gradually decreased from the initial 8.0 mm^2 during the first 5 minute to 1 mm^2 after the week 10. Table 2 shows that, when the injected substance was blood with corticosteroids, the coagulum gradually decreased from the initial 7.5 mm^2 during the first week, to 3 mm^2 during the sixth week, to become immeasurable in the week 9. We could not explain what happened at week 4, when coagulum paradoxically increased about 7.0 mm^2 and then gradually decreased to become immeasurable in the week 9. Fibrinolysis as a function of coagulum reduction, in cases when blood was injected, had a form of the second order polynomial: Yt=0.0535X2-1.1591X+6.9746, with a trend error SYt=0.77, which could be used for interpolation and extrapolation of data in the cases having the characteristics of the analyzed specimen. The correlation of the coagulum size and time in weeks, in cases when the blood was injected (Table 1) was highly significant (r=0.95; p<0.001). Explanatory factor of time impact (in weeks) on the reduction of the size of the coagulum was 91.2%, while the remaining 8.8% of variability was attributed to other factors. Using the same analysis in cases when blood and corticosteroids were injected (Table 2), the following for B+CS was obtained (94.6% to 5.4%).

The cellular response to vitreous B and B+CS was analyzed by counting and establishing an average cell number seen within 3 visual fields at characteristic spots, using large magnification (400×). *De facto,* the center of coagulum, its periphery, and locations adjacent to ciliary body and retina were analyzed, by counting the polymorphonuclear (PMN) cells, mononuclear cells, macrophages, as well as fibroblasts and plasma cells. Tables 3 and 4 show the results of examination of coagulum center, coagulum periphery, area adjacent to ciliary body and area adjacent to retina in cases when B and B+CS were injected. Table 3 shows cellular appearance by number and type of cells in the center of coagulum when B was injected, when there were 3 PMN cells, 1 mononuclear cell, and no macrophages after five minutes of blood injection. Table 4 shows the results of examination of coagulum center when blood and corticosteroid were injected, where we can see situation similar to that seen in cases when blood alone was injected. Herein, more than 10 PMN, mononuclear cells, and macrophages in the week 4, as well as one mononuclear leukocyte during the week 10 can be noted.



Graph 1. Blood as an injectant

Each bar represents the eye of one rabbit, while its peak represents the end of examination period when the rabbit was eutanized.



Graph 2. Blood and corticosteroids as an injectant

Each bar represents the eye of one rabbit while their peaks represent the end of examination period when the rabbit was eutanized

Table 1. Fibrinolysis in function of coagulum reduction – blood as an injectant

Time	Size of the coagulum (mm^2)
5 minutes	8
Week 1	5.5
Week 2	4.5
Week 3	3
Week 4	Not measured
Week 6	2
Week 9	2
Week 10	1
Week 12	0

Table 2. Fibrinolysis in function of coagulum reduction – blood and corticosteroids as an injectant

Time (week)	Size of the coagulum (mm^2)
1	7.5
2	5
3	4
4	7
6	3
9	0
10	0
12	0

doi: 10.2298/SARH1312732V

Table 3. Cell number and type according to intervals of time in cases where blood was injected

Number of the sample	Time intervals	Coagulum center			Coagulum periphery			Near the ciliary body			Near the retina		
		PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages
1	5′	3	1	0	0	0	0	0	0	0	0	0	0
2	Week 1	0	0	0	3	3	1	0	0	0	1	1	10
3	Week 2	1	1	0	1	4	3	0	10	3	0	5	10
4	Week 3	0	0	0	0	2	1	0	2	2	0	0	10
5	Week 4	0	0	0	2	2	1	0	0	0	0	0	0
6	Week 6	Can not see			Can not see			0	3	0	0	3	0
7	Week 9	0	0	0	0	3	3	0	0	0	0	0	0
8	Week 10	Can not see			Can not see			1	0	0	Can not see		
9	Week 12	0	0	0	3	3	0	0	1	0	0	3	1

An average number of cells in 3 fields of view of high magnification (×400).

Table 4. Cell number and type according to intervals of time in cases where blood + corticosteroid were injected

Number of the sample	Time intervals (week)	Coagulum center			Coagulum periphery			Near the ciliary body			Near the retina		
		PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages	PMN cells	Mononuclear cells	Macrophages
1	1	0	0	0	1	1	0	5	10	10	0	0	0
2	2	0	0	0	0	1	1	0	0	0	0	0	0
3	4	1	1	10	1	2	2	0	0	0	0	0	1
4	6	0	0	0	0	3	Fibro	0	3	Fibro	0	0	0
5	9	0	0	0	0	0	3	0	0	0	0	0	3
6	10	0	1	0	0	1	3	0	3	Fibro	0	3	0
7	12	0	0	0	0	0	3	0	0	0	0	0	0

Average number of cells into 3 field of view of high magnification (×400).
Fibro – fibroblast cells

DISCUSSION

This type of experiment which we have done is very difficult to perform. But, every experiment has many elements of variability. Findings may vary significantly from section to section of tissue in our experiment and the results have to be taken with a grain of salt. The findings of other authors are more or less unreliable, too. Results that we need cannot be obtained from human materials alone. Clinically, we can follow vitreous hemorrhage in humans, but histologically it is almost impossible to get human vitreous samples. We hope that our histological findings obtained from animals will give some insight into better clinical understanding of vitreous hemorrhage. The inflammatory cell response can be followed by counting radio-labeled cells. Echo may be used as a supplement method to indirect ophthalmoscopy to follow the clinical stages of vitreous hemorrhage. These methods were not available to us during the time of our experimental work. Nevertheless, in our opinion, these results are the best that can be obtained from the methodological technique used in our experimental work.

If the injected substance, as mentioned above, was always the same amount of autologous blood (0.2 ml), we had an opportunity to determine the area of the coagulum by measuring its length and width using the gross-anatomical specimens (this can be also seen in the anatomical photographs), and therefore gaining the comparable parameter for the analysis in our experiment. Table 1 shows that, when blood was injected, the size of coagulum started to decrease five minutes after the blood injection, from 8 mm^2 to negligible size of 1 mm^2 after the week 10.

The results of comparison of opacity, between the group with blood only and the group with blood and CS, suggest that the corticosteroids were significantly superior in vitreous clearance rate. The results were obtained using the Kaplan-Meier test; they suggested that the probability for all vitreous bodies to clear before the fifth week was 100% in cases where blood was injected. The probability of 100% for all vitreous specimens to clear was reached in week 4 in cases when B+CS were injected. Similar course of vitreous hemorrhage may be noticed when comparing the Graphs 1 and 2. Graph 1 represents the group of rabbits treated with blood only, and Graph 2 group of rabbits treated with blood and corticosteroid, as well as clearance in presence of corticosteroids compared to blood only ($p<0.05$). The probability of complete vitreous clearance when only blood was injected was 60% in the fourth week, and in case of both blood and corticosteroid injection, the probability was 100% in the fourth week. Five minutes after injection, the average number of cells seen on 3 visual fields, within the center of coagulum, was 3, for PMN cells, 1 for mononuclear cells, and none for macrophages. This suggests that these cells did not migrate into the vitreous, but were introduced by injection.

In the first week of assessing cellular dynamics after blood injection, no PMN cell, no mononuclear cell, and no macrophages were observed. In the second week of assessing cellular dynamics after blood injection, 1 PMN cell, 1 mononuclear cell, and no macrophages were observed. Until week 12 of the experiment, no cells within the center of coagulum were observed (Table 3). The sections of fibrin clots and their central areas, in case where blood and corticosteroid were injected into the vitreous (Table 4), showed no PMN cells, mononuclear cells, or macrophages during the first week. In the fourth week, PMN, mononuclear, and macrophage mass became evident, followed by no cells in the sixth week. Week 9 was characterized by the absence of any cells. In the week 10, there were 0 PMN, 1 mononuclear cell, and no macrophages in 3 visual fields, when large magnification was used to examine the central coagulum. In the week 12, no cells of any type were observed.

Considering the fact that coagulum in rabbit skin is removed within week or two [3] in abundant presence of PMN cells, and that the same process in vitreous takes 6-9 weeks in presence of an extremely small number of PMN cells, we may conclude that the reason for slow fibrin elimination is complete absence or modest amount of PMN cells, which is compatible with the results of other authors [10-13]. The absence of significant PMN cellular reaction, which, in fact, facilitates fibrin disintegration, results in slow vitreous fibrin elimination. Furthermore, this leads to fibrin degradation products, which are known to stimulate PMN cellular migration [14, 15, 16].

CONCLUSION

Analyzing the obtained results from our experimental study, whose purpose was to demonstrate normal intravitreous hemorrhage evolution over the successive periods of time, as well as to assess any possible modifications caused by corticosteroids, we can conclude that normal intravitreous hemorrhage evolution differs from other types of hemorrhage affecting the tissues other than eye, by following characteristics: slow fibrinolysis, and the absence of an early polymorphonuclear cellular response to vitreous hemorrhage.

Vitreous blood clearance will start one week earlier in the presence of corticosteroids compared to the clearance when no corticosteroids are administered.

Vitreous response to presence of coagulum is characterized by the absence of inflammatory cells penetration into the coagulum, and by persistence of coagulum until week 4.

The loss of coagulum fibrin network is not observed before week 4-6 after the injection of blood. Accordingly, it is reasonable to wait at least 6 weeks for spontaneous clearance of vitreous hemorrhage.

doi: 10.2298/SARH1312732V

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Природни ток експерименталне витреалне хеморагије и утицај кортикостероида на њен ток

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КРАТАК САДРЖАЈ

Увод Ток и прогнозу витреалног крварења тешко је предвидети упркос подацима доступним у објављеним експерименталним и клиничким студијама.

Циљ рада Циљ рада је био да се прати ток и сагледа прогноза витреалног крварења.

Методе рада Експеримент је изведен на 19 чинчила кунића (*Oryctolagus cuniculus*) оба пола (38 очију), тежине 2500–3000 грама, којима је у очи убризгана аутологна крв из ивичне вене ува (прва група животиња), односно аутологна крв и кортикостероидни раствор (друга група). Праћен је и анализиран ток витреалне хеморагије.

Резултати Резултати експеримента су показали да је ћелијска реакција на витреално крварење различита у поређењу с крварењима у другим ткивима, првенствено због изостанка ране полиморфонуклеарне ћелијске реакције. Број ћелија унутар витреалног простора био је веома мали.

Закључак Витреална хеморагија има специфичан ток праћен малим бројем ћелија (углавном полиморфонуклеарним ћелијама). То је главни разлог зашто се интравитреално крварење ресорбује веома споро (просечно, између шест и девет недеља). Кортикостероиди имају умерен утицај на убрзање апсорпције витреалне хеморагије.

Кључне речи: витреална хеморагија; природни ток; кортикостероиди; зечеви

Примљен • Received: 05/03/2012 **Прихваћен • Accepted:** 30/05/2012