Non-occlusive retinal vascular inflammation and role of red blood cell deformability in birdshot chorioretinopathy

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Abstract:

Purpose: To investigate differences in red blood cell (RBC) deformability between birdshot chorioretinopathy (BCR) subjects and matched controls, and to postulate its relationship with lack of vascular occlusion in BCR.

Methods: In a single center, prospective, non-randomized mechanistic study, blood samples were collected from 8 healthy controls and 9 BCR patients and subjected to biochemical and hematological tests as well as RBC indices assessment using dual-beam optical tweezers.

Results: The mean age of the controls was 52.37±10.70 years and BCR patients was 53.44±12.39 years. Initial cell size (Io) for the controls was 8.48±0.25 μm and 8.87±0.31μm for BCR RBCs (p=0.014). The deformability index (DI) for the controls was 0.066±0.02 and that for BCR RBCs was 0.063±0.03 (p=0.441).

Conclusion: There was no statistically significant difference in DI between RBCs from BCR and healthy controls. This may explain the rare occurrence of retinal vascular occlusion despite the underlying vasculitic pathophysiology of BCR.

Introduction:

Birdshot chorioretinopathy (BCR), or vitiliginous chorioretinitis, is an uncommon, chronic, recurrent ocular inflammatory disease, which may have been first described as early as in 1949.[1] BCR occurs more commonly in white individuals who are otherwise healthy, in females more than males, affects those in the fourth to sixth decade of life and is strongly associated with HLA-A29.[2,3] BCR is not known to be associated with any systemic disease.[4] In the eye, BCR affects particularly the posterior segment, involving the choroid and retinal vessels.[2]

The clinical manifestations of BCR include multiple choroidal hypopigmented lesions, vitritis, vasculitis, macular edema and less commonly choroidal neovascularization.[2,3,5-7] Macular edema and macular atrophy have been shown to be the 2 most common causes of poor vision in BCR patients.[8,9] While thrombosis is known to be closely associated with other vasculitic diseases such as Behçet's disease, systemic lupus erythematosus and sarcoidosis[10-12], BCR appears to be an exception, with retinal vascular occlusion understood to be a rare occurrence in this disease.[6,13]

Hemorrheological characteristics, among other factors, may influence thrombosis in the context of vasculitic disease. RBC length, morphology and deformability in particular, have been studied to a limited extent in other ocular vasculitic conditions such as Behcet's disease.[14] There is vascular occlusion in some of the retinal diseases involving the posterior segment (Behcet's disease, tubercular retinal vasculitis, central retinal vein occlusion), however it has not been commonly reported in patients with BCR.[13] The overall pathophysiology and retinal vascular complications of BCR are still poorly understood.[3,15] To the knowledge of the authors, there are no published studies to date which have examined the possible reasons for non-vascular occlusion

and role of RBC biomechanics in BCR. It will be quite interesting to explore the relationship between biophysical properties of the blood and establish its relationship with vascular non occlusion in BCR.

Optical methods for deforming RBCs using either conventional optical tweezers[16-18] or the "optical stretcher" method[19] have proved to be a sensitive tool for detecting changes in cell mechanical properties. In our earlier study, we have studied the biomechanics of RBCs in potential vascular occlusive disease i.e., diabetes mellitus (DM) and diabetic retinopathy (DR) using optical tweezers technique by direct application of the tweezers to the cell membrane of RBCs.[20]

The primary objective of this study was to investigate the biophysical properties of the RBC using the dual-trap optical tweezers method and to postulate hypothesis for non-occlusive retinal vasculitis in patients with BCR. The secondary objective was to analyze the biochemical and hematological parameters affecting RBC morphology and deformability in BCR.

Material and Methods:

This study was conducted as per the declaration of tenets of Helsinki. Ethics board approval was obtained by NRES Committee, West Midlands-Coventry and Warwick and Moorfields Eye Hospital Ethics Committee (14/WM/1038). The study was conducted between August 2014 and July 2015. Nine patients with BCR undergoing treatment and 8 age and gender-matched healthy controls were recruited after obtaining the informed consent. Subjects (patients and controls) with associated systemic diseases like diabetes, hyperlipidemia and on medications affecting the blood rheology and smokers were excluded from the recruitment under this study. Details about the demographics and personal history were recorded on a pre-coded data information sheet. Patients were on systemic anti-inflammatory drugs for varying

duration and that confounding variable was not adjusted in this study; though there is no published literature on this drugs affecting rheology of the blood by virtue of affecting RBC deformability.

Similar protocol to our earlier published report on deformability of diabetic RBCs was followed.[20] A total of 20ml blood from each subject was collected for hematological and biochemical tests, with 3ml of the blood collected in a spray-coated K2-EDTA (plastic) BD® vacutainer tube with BD hemogard™ closure for assessment of RBC deformability. Assessment of RBC deformability was made using the same dual optical tweezers apparatus described in detail in our previous study on diabetic subjects.[20,21] Briefly, the dual optical tweezers set up was made by splitting and recombining a single Nd:YAG laser beam using polarization optics as shown in **Figure 1.**[21] Both beams were injected into the fluorescence port of an inverted microscope (Zeiss Axiovert 200) and focused by a high numerical aperture (NA = 1.3, oil immersion) x100 objective (**Figure 1**) on the RBCs. For a small separation between the laser foci, RBCs are trapped in the orientation shown in the inset of **Figure 1,** where the black dots represent the positions of the laser foci, i.e. they are observed "side-on **(Figure 2A).** The cells were stretched using the same protocol as reported previously **(Figure 2B)**, that is by slowly increasing the trap separation using the galvanometer mirrors to stretch them, then releasing the applied tension by quickly jumping one of the laser foci to a large distance away while the cells relaxed to their unstretched lengths. The cells were then recaptured, and then brought back to their initial state by returning the laser foci to their initial separation.

An experimental sequence consisted of 5-10 of the above cycles per cell. Each cell was subjected to 3 x 5-10 cycles in a time of less than 5 minutes and for each sample we attempted to assess 10 random cells. Measurement of the initial transverse length

and final stretched length of the RBC was done using custom-written image analysis software in Matlab™ **(Figure 2C)** (TS/CR).

Deformability index (DI): DI was computed using the following simple formula:

DI = [Final stretched length of RBC (Imax) – Initial unstretched length of RBC (Io)] ÷ Initial unstretched length of RBC (Io)

Statistical analysis: The data was analysed using STATA/IC version 13.0 (Stata Corp, College Station, TX, USA). The qualitative variables were expressed as percentages and quantitative variables as mean ± standard deviation for normally distributed variables. Mean RBC indices were calculated for each individual subject. Comparison of the mean RBC indices between two groups (control group and BCR group) was done by Wilcoxon rank-sum test. Spearman's rho correlation coefficient with Bonferroni-adjusted p-values was performed to study the correlation between the hematological and biochemical variables and Io and DI. Results were considered statistically significant if p was <0.05.

Results:

A total of 8 control subjects (control group) [mean age: 52.37±10.70 (40-69) years, 95% CI: 43.42, 61.32; 4 (50%) males] and 9 BCR subjects (study group) [mean age: 53.44±12.39 (32-71) years, 95% CI: 43.92, 62.96; 6 (66.67%) females] were recruited for the study. The **complete blood count**, liver function test, cholesterol profile, renal function test and coagulation profile were compared between the two groups and did not show any statistical significant difference between two groups except for total neutrophil count and chloride levels in the blood. Neutrophil count was elevated while chloride level was lower in the patient group (**Supplementary Table 1**).

With 60% laser strength, we were able to capture 80 frames per second. There was slippage of the cell from the trap if same cell was trapped for longer than 2 minutes. Other observations noted were the intermittent rotation of the cells while being trapped within the laser.

A summary of the RBC deformability indices for each subject of both the groups in **supplementary Table 2**. The average Io of RBCs for control group was 8.48±0.25 (8.03- 9.25) and 8.87±0.31 (8.18-9.73) μm for BCR RBCs (p=0.014, Wilcoxon rank-sum test) (**supplementary Table 3**). Average Imax for RBCs was 9.04±0.17 (8.51-9.65) μm for the control group and 9.42±0.26 (8.85-10.71) μm for the BCR group (p=0.003, Wilcoxon rank-sum test) (**supplementary Table 3**). **Figure 3** represents the scatter plot for all the cell cycles for the Io and Imax using linear fit models for the control and BCR group. The mean difference between the Imax from that of the Io was computed for both the control (0.55±0.17, 0.15-1.07) and study groups (0.55±0.26, 0.11-2.23) and revealed no statistically significant difference (p=0.36, Wilcoxon rank-sum test). **Figure 4** represents the Kernel density plots for Io and Imax of RBCs for both the control and study groups. The DI for control group was 0.066±0.02 (0.017-0.131) and that for the BCR RBCs was 0.063±0.03 (0.012-0.270) (p=0.441). **Figure 5** represents the scatter plot for the DI for both the study and control groups in comparison with the Io. The correlation between all the hematological and biochemical parameters with Io and DI did not reveal any statistical significance.

Discussion:

There was statistically significant difference in the basal cell size and deformed cell size of RBCs, but there was no significant difference in the deformability of RBCs for patients with BCR. The contributory influence of hemorheological alterations on

thrombosis or vascular occlusion is an important one. These include alterations to the plasma viscosity, hematocrit, as well as RBC characteristics including RBC aggregation and RBC deformability.[14] These RBC characteristics are likely to affect overall blood viscosity more than other factors such as plasma viscosity, hematocrit and other blood cells. RBC deformability in particular, is a significant but often understated hematological parameter which affects microcirculation and is an important factor in RBC aggregation and thrombosis.[14] The more deformable the RBCs, the lesser the likelihood of an increase in blood viscosity.[22]

Various factors within the framework of Virchow's triad may contribute to the occurrence of vascular thrombosis: (1) vasculitis, (2) hemorheological changes, and/or (3) hemodynamic changes of blood flow.[23] Klein and Olwin have also postulated similar mechanisms for central retinal vein occlusion.[3] In general, ocular vasculitic conditions are associated with thrombosis and vascular occlusion, accounting for up to 5% of branch vein occlusions.[24,25] Vascular occlusion as complication of vasculitis has been shown to result in significant ocular morbidity, visual loss and decreased vision-specific quality of life.[26]

RBC deformability and characteristics have been studied in other ocular vasculitic diseases such as Behçet's disease, where thrombosis has been attributed to various factors such as vasculitis of the vasa vasorum and hemorheological alterations. Among the hemorheological alterations, abnormal RBC deformability specifically has been observed in patients with active Behçet's disease [14] but not in patients with stable or inactive Behcet's disease.[27] Other factors such as the mean platelet volume, however, have not been shown to be associated with thrombosis or posterior uveitis in this disease [28] while other studies have shown altered cholesterol metabolism to be implicated instead. [29]

However, with the exception of Behçet's disease, the association between RBC characteristics and thrombotic events in other ocular vasculitic diseases has not been extensively studied. The current understanding of BCR in particular, would benefit from further study of this aspect of the disease.

Despite its underlying vasculitic pathophysiology and the high prevalence of retinal vasculitis in active disease[7], BCR is not reportedly associated with thrombosis and vascular occlusion compared to other vasculitic diseases. Systemic lupus erythematosus [12] and Behcet's disease[25] have been shown to have a significantly increased risk of retinal vessel occlusions, where both arterial and venous occlusions have been observed. Sarcoidosis, while not as extensively reported as the above conditions, has also been reported to be associated with retinal vein occlusions.[30] However, the association with thrombosis and retinal vein occlusions appears to be much weaker in BCR. In a review of 102 eyes with BCR in 1988, Priem et al found venous occlusions to be present in only 3 subjects.[6] In another study, a cross-sectional examination of 80 BCR subjects did not appear to identify any subject with retinal occlusive disease.[31] Cases of central retinal vein occlusion in BCR have only been anecdotally reported.[13]

Our findings of normal RBC deformability characteristics in BCR despite the significant differences in the RBC cell size may account, in part, for the apparent absence of increased thrombotic tendency in this disease condition, despite its underlying vasculitic pathophysiology. However, it is possible that the rare occurrence of vessel occlusion in BCR is also attributable to other factors.

Studies into the retinal vasculature and caliber alterations in BCR have revealed characteristic findings, which may contribute to further understanding in this area. It has been demonstrated that various types of vasculitis (including BCR) alters the

caliber of retinal vessels, affecting the arterioles more than venules.[32] While this may explain the lack of association between BCR and specifically venous occlusions, it does not sufficiently account for the weaker association between BCR and retinal thrombosis compared to other vasculitic diseases. However, of note, in an OCT retinal angiography study of 8 eyes by de Carlo et al [15], BCR eyes demonstrated increased inter-capillary space compared to normal subjects implicating reduced capillary density index or loss of capillaris. It has also been shown that although retinal vasculitis typically characterizes the disease process, BCR manifests more often as arteriolar narrowing rather than retinal vascular hemorrhage or exudation.[6,33] It has yet to be studied if these observations influence the thrombotic tendency of retinal vessels in BCR.

The hemodynamics observed in BCR may also affect the lack of thrombotic tendency of this disease. "Quenching" is a fluorescein angiographic phenomenon that is uniquely associated with BCR and describes the rapid emptying of dye from retinal vessels, accompanied by delayed circulatory and arteriovenous transit times.[34] It is a phenomenon not observed in other vasculitic diseases and further studies are required to understand its correlation with thrombotic tendency.

Finally, differences in vessel wall inflammation at a histopathological level between BCR and other vasculitic diseases may help to account for the different thrombotic tendencies among these disease conditions. Gaudio et al previously demonstrated the presence of multiple foci of lymphocytes at different levels of the choroid and around retinal blood vessels in an eye with BCR examined at autopsy.[35] Unfortunately, there is at present a lack of data regarding characteristic retinal vasculature histopathologic findings in BCR.

The optical tweezers technique is a relatively established technique to assess RBC deformability. [16-18] Numerous multiple trap optical tweezers set up have been proposed to measure the elastic properties of the cell. Optical force has either been applied using microbeads attached to the cells as handles [36], or by direct application of laser beam to the cells. [37]

The authors acknowledge the limitations of this study. Firstly, the sample size of this study is limited, with only 9 BCR subjects and 8 matched controls. Nonetheless, this study should serve as a useful precursor for further extensive hemorheological BCR studies with larger sample groups and the utilization of other investigative tools of RBC deformability. RBC deformability, if applied in the wider context of other diseases, may also pave the way for the development of diagnostic tools for these conditions. Secondly, although it is understood that smoking, hypertension and other metabolic diseases may affect RBC deformability and characteristics, the cases and controls in this study were not matched for these potential confounding factors. Thirdly, data was not collected regarding the treatment administered to the BCR subjects. It is acknowledged that various medications, systemic or otherwise, may influence the results of the study.

In conclusion, this pilot study has demonstrated the presence of statistically significant difference for RBC cell size but no differences in cell deformability in BCR subjects as compared to normal population. This may account, in part, for the apparently low risk of increased thrombotic tendency and the rare occurrence of retinal vessel occlusion in BCR, despite its underlying vasculitic pathophysiology. The findings of this study should complement other factors such as retinal vasculature changes and hemodynamic alterations, which are also known to influence thrombotic tendency in vasculitic diseases. We aim to perform similar studies in the near future

on patients with Behcet's disease, presumed tubercular retinal vasculitis, central retinal vein occlusion and explore the causal relationship between vascular occlusion and inflammatory diseases involving the eye.

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Figure Legends:

Figure 1: Experimental apparatus. The laser beam is split into two and recombined using the polarization optics shown. The galvanometer steering mirrors are used to control the relative positions of the laser foci be steering one of the beams. The inset shows an image of an optically trapped red blood cell with the detected boundary as determined by our image analysis software shown. Also shown are the positions of the laser foci (black dots) that trap the cell. The scale bar is 5 μm.

Figure 2: Side on images of RBC under dual beam optical tweezers set up. 2a: Unstretched RBC: Side on view of an unstretched RBC (scale bar: 5μm). **2b:** Stretched RBC: Side on view of a stretched RBC (scale bar: 5μm). **2c:** RBC boundaries demarcated – continuous edges representing unstretched RBC, broken edges representing stretched RBC, black dots indicate laser spots at the start and white dots indicate the laser spot position at the time of maximum stretch.

Figure 3: Scatter plot representing the linear relationship between initial unstretched length of RBCs with final stretched length of RBCs for both the control and BCR groups.

Figure 4: Kernel Density Plots for Initial and Stretched cell sizes for the control and BCR groups.

Figure 5: Scatter plot showing the difference between linear fit lines for control and study groups for deformability index and initial RBC cell sizes.