• Clinical Research •

Vitreous microparticles contain apoptotic signals suggesting a diabetic vitreopathy

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Received: 2021-02-19 Accepted: 2021-09-03

Abstract

● **AIM:** To evaluate differences in microparticle profiles in vitreous samples between diabetic and non-diabetic eyes undergoing vitrectomy.

● **METHODS:** Un-masked cross-sectional series of 34 eyes undergoing vitrectomy. Vitreous specimens were collected and processed to evaluate for membrane integrity (DAPI), apoptosis (Annexin-V), and endothelial-cell origin (V-Cadherin). A BD LSR II flow cytometer was used for analysis and standardized sub-micron-sized beads were used for size comparison.

● **RESULTS:** Thirty-four specimens underwent analysis. Greater levels of Annexin-V were found on microparticles from specimens in which blood had entered the vitreous (n=12) compared to those without blood (n=22; 52.3%±30.7% *vs* 19.6%±27.2%, P=0.002). Patients with diabetes having surgery with hemorrhage (n=7) had greater expression of Annexin-V than those without hemorrhage (n=8; 62.1%±31.7% *vs* 18.9%±20.9%, P=0.009). However, in patients with non-diabetic vitreous hemorrhage, the level of Annexin-V expression was not significantly different compared to other disease processes (38.6%±25.7%, n=5 *vs* 20.0%±30.9%, n=14, P=0.087).

● **CONCLUSION:** Increased expression of the apoptotic marker, Annexin-V is detected on vitreous microparticles in diabetes-related vitreous hemorrhage. When evaluating vitreous hemorrhage in patients without diabetes, the apoptotic signal is not significantly different. Vitrectomy in patients with diabetes, and improvement in visual outcomes, may be related to the removal of a serum-derived, pro-apoptotic vitreous. Further investigation is warranted in order to identify the molecular characteristics of microparticles that regulate disease.

● **KEYWORDS:** cell-derived microparticles; vitrectomy; vitreous; diabetes mellitus; apoptosis
DOI:10.18240/ijo.2022.01.14

Citation: Sultan H, Rajagopal R, Rao PK, Piggott KD, Paley MA, Hassman LM, Li AS, Marshall B, Apte RS. Vitreous microparticles contain apoptotic signals suggesting a diabetic vitreopathy. *Int J Ophthalmol* 2022;15(1):89-97

INTRODUCTION

The vitreous body fills the posterior segment of the eye and is composed primarily of collagen and hyaluronic acid. These extracellular matrix-molecules are synthesized by hyalocytes, the resident cells of the vitreous[1]. Many disease processes will manifest in the vitreous; for example, uveitis may present secondarily in the form of vitreous cells, or proliferative diabetic retinopathy may present in the form of vitreous hemorrhage or tractional retinal detachment. The vitreous itself, however, has not been evaluated as a primary source for disease pathogenesis. To begin elucidating the role of the vitreous in various diseases, membrane-bound extracellular vesicles released by hyalocytes have been examined to explore their potential role in disease pathogenesis.

Microparticles (MPs) are small vesicles bound by a lipid bilayer that pinch off the surface of a cell membrane. The size of MPs range from 0.03-1.0 μm[2]. Exosomes, or membrane-bound vesicles released by a cell, are formed in the Golgi apparatus or lysosomes, and range in size from 0.03-0.1 μm in diameter; while apoptotic bodies tend to be larger, measuring at the 0.05-2.0 μm range. MPs consist of fluid and carry a variety of lipid, protein, and nucleic acid cargo including mRNA and

micro-RNAs (miRNA) which can have downstream effects at remote target locations. Barutta *et al*[3] described differences in serum miRNAs within extracellular vesicles between patients with diabetes, and found that patients with significantly lower levels of miR-126 within serum extracellular vesicles (EVs) were found to have microvascular complications including diabetic nephropathy. The function of MPs both systemically and within the eye remains an area of intense investigation.

There is clear evidence that the pathogenesis of diabetic macular edema and diabetic retinopathy stem from pericyte dysfunction, vascular leakage, and a violation of the blood-retinal-barrier[4-5]. Extravasation of serum contents into the retina and vitreous may alter the microenvironment of the posterior segment which may lead to exacerbation of retinopathy. Other measures of systemic hyperpermeability such as urinary albumin excretion rate was found to be directly correlated to optical coherence tomography (OCT) macular thickness in patients with diabetes[6]. As such, the presence of serum contents in the posterior segment as a result of retinal vascular hyperpermeability may play a role in disease pathogenesis.

There is convincing evidence that vitreous components play a role in disease pathogenesis relevant to therapy. Physicians use anti-vascular endothelial growth factor (VEGF) agents to treat diverse retinal vascular diseases including diabetic retinopathy and age-related macular degeneration[7-8] to target elevated levels of VEGF in the posterior segment of the eye. The ability of an intravitreal injection to improve visual outcomes in these patients has revolutionized the way we treat these diseases[9]. As such, the vitreous can serve as a reservoir for disease-associated or causal molecules that could potentially be future therapeutic targets or biomarkers.

Further clinical and basic-science investigations are evaluating the role of the vitreous in various diseases. The Diabetic Retinopathy Clinical Research (DRCR) Network Protocol D evaluated the role of primary vitrectomy in the treatment of diabetic macular edema[10]. In an era when 20-gauge vitrectomy was commonplace and without a consensus regarding the peeling of macular membranes, the outcomes for Protocol D demonstrated that even in patients that had failed conventional therapy for macular edema, up to half of study participants had some short-term improvement in visual acuity and in OCT thickness. This study, however, was a small cohort study that was not designed to address the role of the vitreous in diabetic disease. Protocol AB, which compares prompt vitrectomy to anti-VEGF treatment, will evaluate the role of small-gauge vitrectomy as a primary treatment modality for proliferative diabetic retinopathy. Follow-up for the study is currently underway. The DRIVE-UK study evaluated outcomes following vitrectomy in patients with diabetic eye disease[11]. Patients with mild vitreous hemorrhage treated with vitrectomy had better outcomes than those treated for tractional retinal detachments supporting the concept that the removal of a diseased vitreous may improve future visual outcomes.

Vitreous MPs have been evaluated in a few case series. Gupta *et al*[12] have evaluated extracellular vesicles using a non-formalin fixative in combination with various imaging techniques including nanoparticle tracking analysis and confocal microscopy. Another group has shown greater concentrations of vitreous MPs and proinflammatory cytokines in patients with rhegmatogenous retinal detachments compared to controls[13]. Exosomes are abundantly expressed in the vitreous[14]. The presence of sub-cellular membrane-derived vesicles in the vitreous with different markers suggests that these vesicles play a role in vitreoretinal pathophysiology. The aim of the current study was two-fold: to optimize the protocol for isolating and identifying human vitreous MPs by flow cytometric analysis, and to identify differences in these MPs between patients with and without diabetes.

Markers used in this study include Annexin-V, CD144, and 4',6-diamidino-2-phenylindole (DAPI). Annexin-V binds phosphatidyl-serine which is typically found on the inner leaflet of a lipid bilayer. When cells undergo apoptosis, there is a reversal to the polarity of the lipid bilayer and the phosphatidyl serine residues are externalized. Once externalized, Annexin-V can bind and identify a microparticle as Annexin-V-positive. CD144 (V-cadherin) is an endothelial cell marker. DAPI is a membrane impermeable molecule and can pass through a lipid bilayer if it has been compromised. DAPI positive MPs identified by flow cytometry indicate that the MP membrane has been compromised and DAPI has entered the MP. Once inside the MP, DAPI binds nucleic acids whether DNA or RNA.

SUBJECTS AND METHODS

Ethical Approval This study was approved by the Human Research Protections Office (HRPO) at the Washington University Institutional Review Board (IRB) and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

Vitrectomy Specimens Inclusion criteria included eyes undergoing primary vitrectomy for any disease process except uveitis. A chart review was performed to acquire historical data such as presence and absence of diabetes mellitus and the reason for surgery. The patients underwent standard 3-port trans-conjunctival vitrectomy with sterile precautions. Three 25-gauge trocars were inserted into the eye and the vitrector was inserted into the mid-vitreous cavity. An assistant would aspirate the vitreous into a 3 mL syringe to an appropriate volume prior to the visualization of choroidals or ocular collapse. For the initial 9 patients, cut rates varied between

1000 cuts per minute (cpm) to 7500 cpm. Subsequent to this, future specimens were cut at 1000 cpm. Specimens were transferred into freezer-appropriate 3 mL vials and immediately placed on dry ice and later placed into a -80°C freezer.

Protocol Optimization In order to optimize MP signal for flow cytometry, it was important to titrate parameters to attain the best possible signal. This signal is the ability for the cytometer to identify a single MP, as opposed to two MPs (doublets) that may be stuck together. When a cytometer reads a doublet, the amount of fluorescence emitted by that particle is therefore an aberrant reading since two MPs would have more fluorescence together than a single MP alone and can alter specimen interpretation. Singlets were defined as a population of particles within a 20K-unit difference in side-scatter-width that may span a range of side-scatter-area-levels, while doublets and other multiplets were particles with greater side-scatter-width values. Vitrectomy cut rates were titrated between 1000 and 7500 cpm to identify cut rates with optimal singlet yield.

Specimen Preparation Samples were thawed at 37°C for 5min and transferred to tissue culture plates with Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 2 mg/mL type II collagenase. The tissue culture plate was placed in a 37°C shaker for 1h rotating at 100 rotations per minute. MPs were then collected per recommendations by the International Society of Thrombosis and Hemostasis[15]. In detail, specimens were centrifuged at 2500 g for 15min. The supernatant was aspirated and resuspended in 1 mL of phosphate buffered saline (PBS) and centrifuged again at 2500 g. The supernatant was aspirated and specimens were resuspended in 100 μL of PBS with mouse monoclonal anti-human CD45 antibody conjugated to fluorescein isothiocyanate (FITC, Thermo Fisher) and mouse monoclonal anti-human CD144 (V-cadherin) antibody conjugated to allophycocyanin (APC, Thermo Fisher) for 30min on ice. Specimens were washed with PBS and centrifuged at 2500 g for 15min. The supernatant was aspirated and the pellet resuspended in Annexin-V-binding-media. The specimen was again spun down at 2500 g for 15min. The supernatant was aspirated and the pellet resuspended in 100 μL of Annexin-V-binding media with Annexin-V bound to phycoerythrin (PE) and incubated for 15min at room temperature. Specimens were washed with Annexin-V binding media and resuspended in a final 200 μL of Annexin-V binding media with DAPI prior to flow cytometry. DAPI-control-specimens were permeabilized with 100% ice-cold methanol at 4°C for 20min.

Evaluation of Membrane Integrity To evaluate MP membrane integrity, non-frozen specimens were evaluated per protocol above with incubation only with DAPI. Specimens were divided into two aliquots and the second aliquot was evaluated the subsequent day. Specimens were kept at 4°C overnight and evaluated the subsequent day for DAPI fluorescence.

Flow Cytometric Analysis A BD LSR II flow cytometer was used for analysis. FITC, PE, and APC compensation beads were used as positive controls for each fluorophore. Prior methanol permeabilized specimens served as positive DAPI controls. The forward scatter laser is a 488 nm laser establishing the lower threshold for particle detection just under 0.5 μm. Sub-micron beads ranging from 0.5-2.0 μm were used for size-comparison. Gating was initially focused on obtaining singlet microparticle populations. Subsequent gating was performed on DAPI negative specimens to evaluate specimens with intact lipid bilayers. Further analysis for percentages of CD45, CD144, and Annexin-V positivity were evaluated with histogram or graphical functions. FlowJo 10 (FlowJo for Windows, Version 10, FlowJo LLC) was used for analysis.

Statistical Analysis Statistical analysis was performed using SPSS statistical software (SPSS for Windows, Version 23.0; IBM-SPSS). Significance was defined with $P<0.05$. As this was an exploratory study, sample size calculations were not performed. Continuous data sets between two groups were compared using the Mann-Whitney *U* test and paired samples at two time points were analyzed using the Wilcoxon-Rank-Sum test.

RESULTS

Lower Cut-rates Improve Microparticle Yield In order to obtain the highest yield of MP singlets, vitrectomy cut rates were altered in order to determine the effect on yield. A low cut rate was established at 1000 cpm and higher cut rates at 4000 and 7500 cpm. Due to differences in vitrector manufacturing, cutting mechanisms vary. The Alcon Constellation™ 25-gauge vitrector cuts once per cut: it cuts as the cutting-apparatus moves towards the tip of the vitrector, but not when it moves back towards the handpiece. The DORC Eva™ system, however, cuts twice per cut, once in each direction as the cutter passes away, towards the tip of the vitrector, and again when the cutting-apparatus returns towards the handpiece. Specimens collected by the Eva-vitrectomy system were collected to approximate the effective cut rate of the Constellation-system (therefore a 500 cpm Eva-collected specimen was equivalent to a 1000 cpm Constellation-collected specimen). Specimens obtained at a lower cut rate yielded 92%±5.5% (mean±SD, $n=4$) singlets per specimen whereas those obtained at higher cut rates yielded 66%±12.8% ($n=5$, $P=0.016$; Figure 1). After the analysis of these 9 specimens, subsequent specimens were collected at the 1000 cpm cut-rate and these former specimens were not included in subsequent analysis.

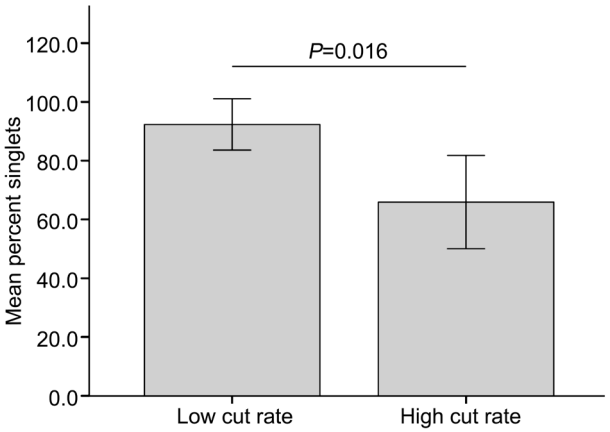

Figure 1 Lower cut-rates improve microparticle yield Mean percent of microparticle singlets within a specimen based on low *vs* high vitrector cut rates. Error bars: 95%CI.

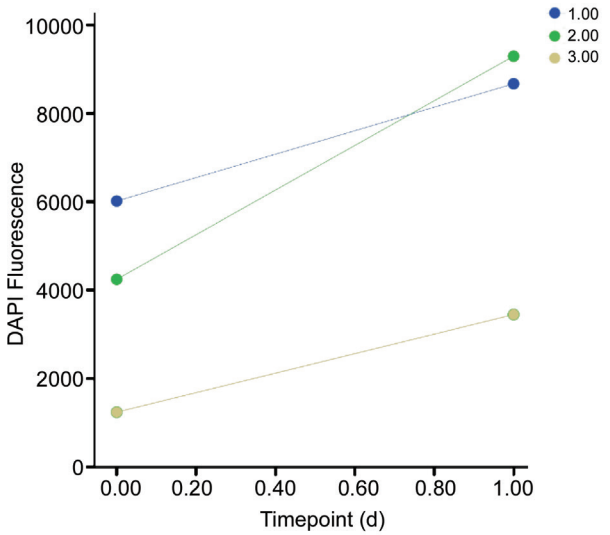

Figure 2 Microparticle membrane integrity diminishes with time DAPI fluorescence at the day of collection compared to DAPI fluorescence the subsequent day. DAPI: 4',6-diamidino-2-phenylindole.

Membrane Integrity Diminishes with Time To evaluate sample stability, membrane integrity was assessed by DAPI exclusion. Fresh MP-specimens were evaluated the day of collection without a freeze-thaw cycle and divided into two aliquots. The first aliquot was examined the same day of collection after incubation with DAPI. The second aliquot was kept at 4°C overnight and evaluated the next day. Specimens evaluated the day of collection demonstrated a lower DAPI fluorescence than the subsequent day, suggesting a decrease in membrane integrity with time. However, difference was not found to be statistically significant in the 3 specimens in which this was performed (*P*=0.106; Figure 2).

Microparticle Singlet Yield Based on Disease Process Certain disease processes were found to inherently have greater percentages of MPs than others among fresh specimens.

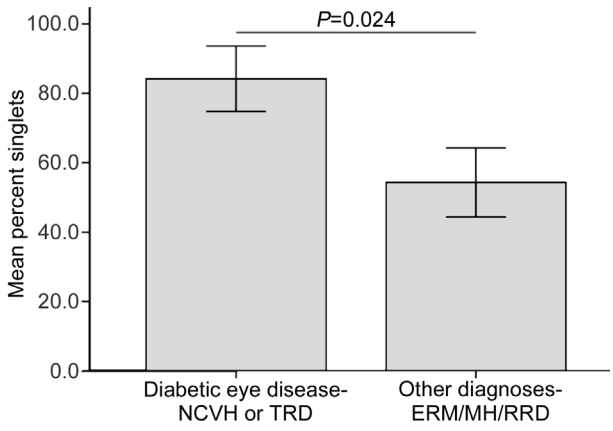

Figure 3 Greater microparticle yield identified in diabetic samples in comparison to other samples Mean singlet percentages between patients undergoing vitrectomy for diabetic complications such as NCVH or TRD compared to other diagnoses, including ERM, MH, and RRD. NCVH: Non-clearing vitreous hemorrhage; TRD: Tractional retinal detachment; ERM: Epiretinal membrane; MH: Macular hole; RRD: Rhegmatogenous retinal detachment; Error bars: 95%CI.

Patients with macular holes or epiretinal membranes were found to have 56%±7.1% (*n*=3) singlets per fresh specimen compared to 88%±8.6% (*n*=6) in patients with diabetic eye disease such as non-clearing vitreous hemorrhage due to proliferative retinopathy or tractional retinal detachment (*P*=0.024; Figure 3).

A representative flow plot is shown in Figure 4A demonstrating a specimen from a macular hole surgery while Figure 4B demonstrates a specimen from a tractional retinal detachment surgery with differences in singlet yields. Once singlets were identified, attention was drawn to DAPI negative populations (MPs with intact membranes), and Annexin-V and CD144 positivity (APC and PE-fluorophores respectively), as shown in Figure 5.

Diabetic Vitreous Expresses Greater Levels of Annexin-V Patient baseline characteristics are shown in Table 1. Patients undergoing vitrectomy for diabetic eye disease such as non-clearing vitreous hemorrhage or tractional retinal detachment have infiltration of whole blood into the vitreous. This alters the MPs in the vitreous in that there are blood-derived MPs in the vitreous in addition to hyalocyte-derived MPs. To explore this, we collected specimens from patients with diabetes who underwent vitrectomies unrelated to their diabetes for conditions such as macular hole repair or rhegmatogenous retinal detachment (*n*=8). We also collected specimens from patients without diabetes who developed non-clearing vitreous hemorrhage from another cause, such as a retinal tear with a torn bridging retinal vessel (*n*=5). A representative flow plot demonstrating Annexin-V positivity between MPs from

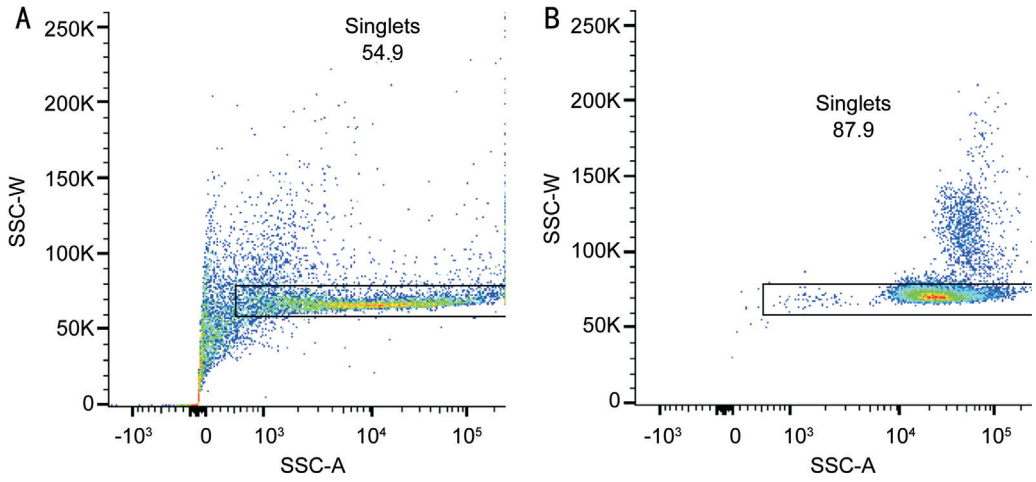

Figure 4 Flow plot comparison of diabetic and non-diabetic samples A: Representative flow plot identifying singlets in a macular hole patient without diabetes; B: Singlet population in a patient with a tractional retinal detachment. MPs above the singlet-gate demonstrate doublets and other multiplets of MPs that may be adhered to one another. MPs: Microparticles.

Table 1 Baseline patient characteristics

Baseline characteristic	*n* (%)
Eyes	34
Sex (male)	19 (56)
Age, mean±SD	64±12.7
Diabetic patients	15 (44.1)
Diabetic patients undergoing surgery not related to diabetes[a]	8 (23.5)
Non-clearing vitreous hemorrhage in a non-diabetic patient	5 (14.7)
Any vitreous hemorrhage (including tractional retinal detachments)	12 (35.3)
Reasons for surgery	
Non-clearing vitreous hemorrhage	9 (26.4)
Epiretinal membrane	6 (17.7)
Macular hole	7 (20.6)
Rhegmatogenous retinal detachment	6 (17.7)
Vitreomacular traction	3 (8.8)
Persistent macular edema	1 (2.9)
Tractional retinal detachment	2 (5.8)

[a]Patients with diabetes that underwent surgery for macular hole or rhegmatogenous retinal detachment. SD: Standard deviation.

a macular hole surgery compared to a diabetic non-clearing vitreous hemorrhage is shown in Figure 6.

The presence of Annexin-V on MPs from patients with and without diabetes was not significantly different overall (39.0%±33.8%, *n*=15 *vs* 24.9%±30.1%, *n*=19, respectively; *P*=0.228). However, MPs from samples with vitreous hemorrhage had greater Annexin-V positivity than MPs from samples without hemorrhage (52.3%±30.7% *vs* 19.6%±27.2%, respectively, *P*=0.002; Figure 7). Amongst patients with diabetes, MPs from hemorrhagic samples had greater Annexin-V positivity compared to those without hemorrhage (62.1%±31.7%, *n*=7 *vs* 18.9%±20.9%, *n*=7, *P*=0.009; Figure 8). In contrast, in samples obtained from patients without diabetes, Annexin-V positivity on MPs in vitreous hemorrhage was not different from other disease processes (38.6%±25.7%, *n*=5 *vs* 20.0%±30.9%, *n*=14, *P*=0.087; Figure 8). Taken together, this data suggests that the presence of Annexin-V positivity, a marker of apoptosis, is a feature of diabetic hemorrhage.

The endothelial marker CD144 or V-cadherin was analyzed and shown to have greater positivity in vitreous with hemorrhage than without hemorrhage (18.2%±22.4%, *n*=7 *vs* 0.8%±1.7%, *n*=12, respectively, *P*=0.007). In the subset of patients with diabetes, this difference was not statistically significant (10.8%±17.5%, *n*=3 *vs* 1.8%±2.5%, *n*=5, *P*=0.786). When evaluating patients without diabetes, those with hemorrhage maintained elevated levels of CD144 compared to other disease processes (23.8%±26.5%, *n*=4, *vs* 0.02%±0.03%, *n*=7, respectively, *P*=0.006; Figure 9). This finding suggests a baseline vascular leakage in non-hemorrhagic diabetic specimens and confirming greater levels of endothelial-derived MPs in specimens with hemorrhage.

DISCUSSION

This study aimed to establish methods to improve signal quality for performing flow cytometry on human vitreous MP populations. In addition, differences in MP profiles between patients with and without diabetes who have undergone vitrectomy were also established during this study.

Isolation and Evaluation Due to their small and variable sizes, many methods have been devised to best extract and isolate exosomes and EVs from various tissues. The centrifugation method used in this study was that recommended by the International Society of Thrombosis and Hemostasis[15]. MP-isolation from vitreous presented an additional challenge compared to serum protocols due to the

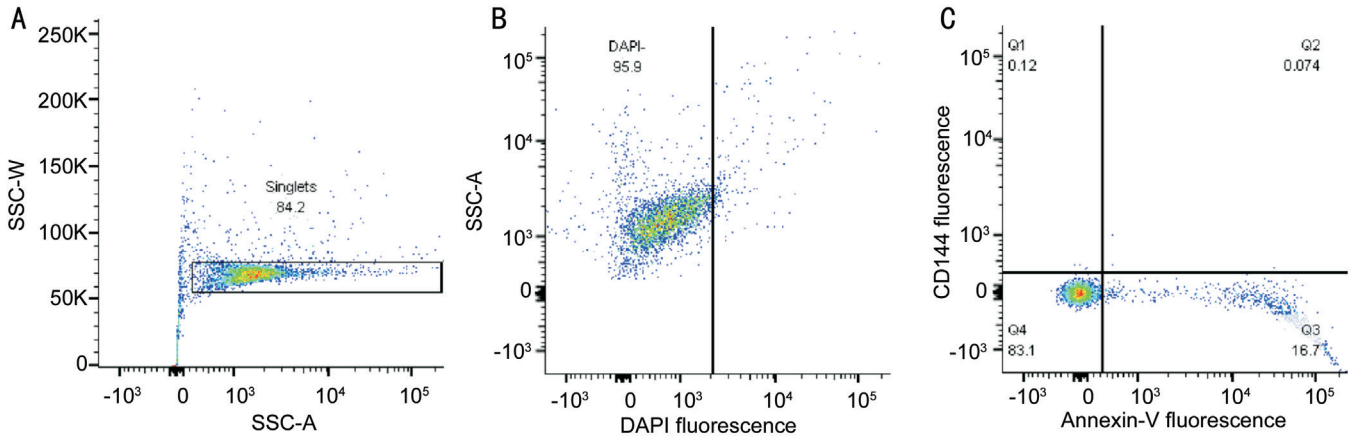

Figure 5 Gating strategy for identifying surface markers of vitreous microparticles A: Identification of singlet MPs on a Side-Scatter-Area (SSC-A) *vs* Side-Scatter-Width (SSC-W)-plot; B: Identification of singlet MPs that are DAPI-negative, indicating intact microparticle membrane structures; C: Identification of DAPI-negative, Annexin-V positive (indicated by the fluorophore phycoerythryin) and CD144 (indicated by the fluorophore allophycocyanin) positive populations. Negative MPs for both Annexin-V and CD144 are on the bottom-left of the diagram. DAPI: 4',6-diamidino-2-phenylindole; MPs: Microparticles.

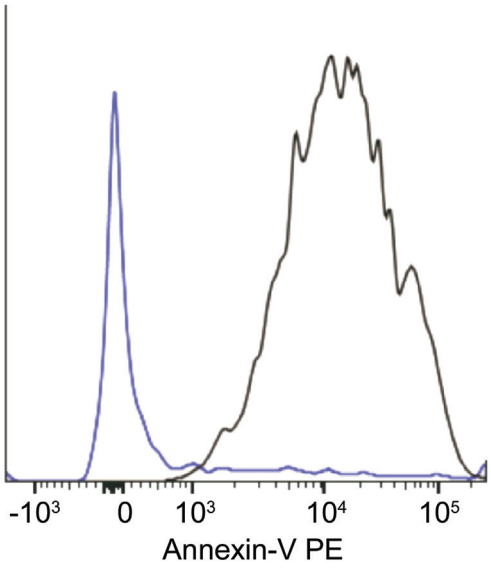

Figure 6 Representative difference in Annexin-V-PE positivity between two specimens The left specimen demonstrates a patient with a non-diabetic macular hole with relatively little Annexin-V positivity, while the specimen right demonstrates a patient with a diabetic non-clearing vitreous hemorrhage with significant Annexin-V-positivity.

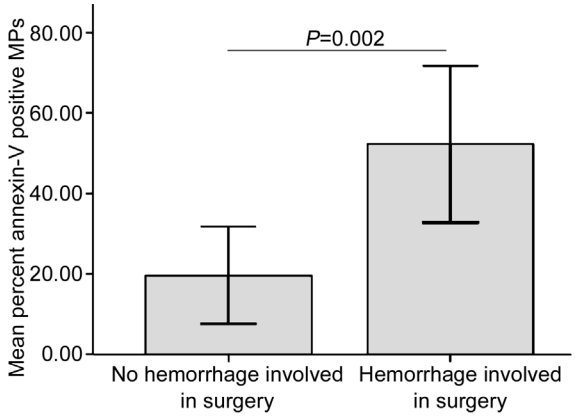

Figure 7 Hemorrhagic vitreous contains greater levels of Annexin-V positive microparticles Mean Annexin-V positive MPs between specimens in which surgery did not involve hemorrhage *vs* those that involved hemorrhage. MPs: Microparticles; Error bars: 95%CI.

presence, in vitreous, of overlying extracellular matrix-tissue. To address this, vitreous specimens were thereby digested with type II collagenase in tissue culture media for 1h prior to centrifugation.

Methods for optimizing MP signal for flow cytometric analysis are also described. Reducing cut rate to 1000 cpm compared to the general maximum cut rates of 7500-8000 cpm may preserve MPs integrity and reduce debris formation which may occur with faster cut rates. We noted only minimal loss of MP membrane integrity during storage at 4°C overnight as evidenced by an increase in DAPI fluorescence on the subsequent day. While the change in membrane integrity was not significantly different in 3 samples, this finding indicates that evaluating fresh specimens is preferable, as overnight storage does degrade specimen quality. Subsequent specimens in this study, however, were evaluated after being frozen.

The signal strength (percent of MP singlets in a sample) was found to be greater in certain diseases such as diabetic non-clearing vitreous hemorrhage and tractional retinal detachment compared to other surgeries such as rhegmatogenous retinal detachment, macular hole surgery, or epiretinal membrane surgery. This may be partly due to blood having entered the vitreous and contamination of vitreous MPs by whole blood MPs. An increase

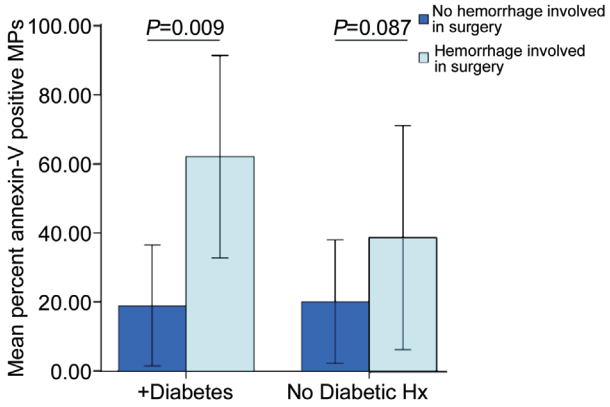

Figure 8 Hemorrhagic, diabetic vitreous contains greater levels of Annexin-V positive microparticles compared to non-diabetic-samples Mean Annexin-V positive MPs between patients with or without diabetes stratified by presence or absence of vitreous hemorrhage. MPs: Microparticles; Error bars: 95%CI.

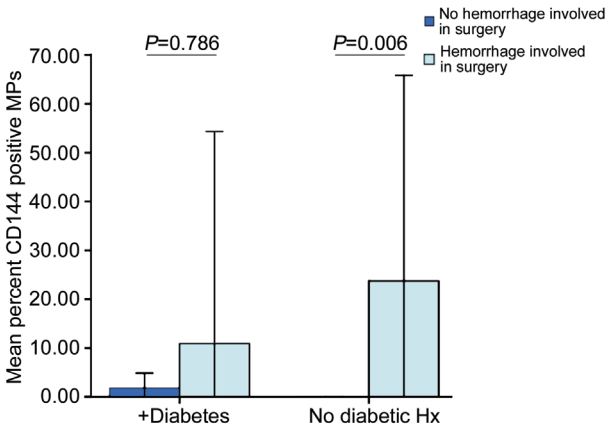

Figure 9 Greater levels of CD144 positive microparticles identified in hemorrhagic samples compared to non-hemorrhagic samples Mean CD144 positive MPs between specimens with blood *vs* specimens without blood stratified by diabetic status. MPs: Microparticles; Error bars: 95%CI.

in total MPs by blood MPs would improve the singlet population of MPs found in the vitreous and thereby signal strength.

Implications of Microparticles in Diabetic Eye Disease EVs and MPs are released by nearly all cell types and play a role in physiologic conditions such as cellular activation[16] and apoptosis[17] to pathologic conditions including malignant transformation[18] and inflammation[19]. The role of vitreous MPs in the pathogenesis of diabetic retinopathy, however, has not been characterized.

Endothelial and pericyte dysfunction play a clear role in the pathogenesis of diabetic retinopathy and the leakage of vascular contents into the retina[4-5,20-21]. Intact tight junctions within retinal endothelium maintain the blood-retina barrier keeping out various antigenic stimuli, macromolecules and serum MPs. Endothelial dysfunction in diabetes, however, can result in the production of pathologic MPs which contribute to the inflammatory and atherogenic response[22] as well as leakage of serum contents into the retina and vitreous. Circulating serum MPs have been evaluated in patients with varying levels of glucose tolerance demonstrating a gradual increase in Annexin-V^+ MPs in the serum of patients with greater glucose intolerance[23].

Patients with vitreous hemorrhage were found to have greater levels of Annexin-V^+ MPs than those without vitreous hemorrhage. This difference, however, was specific to diabetic vitreous hemorrhage, and not vitreous hemorrhage explained by other disease processes such as a torn retinal vessel in the setting of a retinal tear or detachment. Increased Annexin-V positivity in serum MPs have been previously described in patients with diabetes by Giannella *et al*[23]. Extravasation of serum contents, including pro-apoptotic MPs, into the posterior segment may play a role in the progression of diabetic retinopathy. Methods to dampen the extravasation of serum contents may help improve outcomes. One pathway that treats vascular hyper-permeability, the Tie2/Angiopoietin pathway, has been shown in clinical trials to treat diabetic macular edema and age-related macular degeneration and to help improve outcomes[24]. Inhibition of such pathways may reduce the extravasation of these pro-apoptotic markers into the vitreous, however these functional assays have yet to be performed.

Eyes containing vitreous hemorrhage also had greater levels of $CD144^+$ MPs than those without hemorrhage. Chahed *et al*[25] describe increased $CD144^+$ MPs in patients undergoing vitrectomy for proliferative diabetic retinopathy, however this may be secondary to vascular leakage and introduction of serum MPs into the vitreous rather than being pathology related to diabetes. Evidence is provided in this study showing that vitreous hemorrhage even in patients without diabetes contains elevated levels of $CD144^+$ MPs. This is not a surprising finding as most MPs contain markers from their cell of origin; and MPs from the vasculature would contain elevated levels of CD144 which is derived from endothelium.

The presence of an abnormal MP-environment within the vitreous may play a role in the worsening of diabetic retinopathy. The DRCR.net Protocol AB may show how early vitrectomy may compare to anti-VEGF treatment alone, and to see if removal of a diseased vitreous may improve visual outcomes. Vascular leakage and breakdown of the blood-retina-barrier and introduction of serum MPs may contribute to worsening retinopathy. The removal of these diseased MPs may improve outcomes. Vitreomacular adhesions are associated with worse diabetic retinopathy outcomes, supporting the notion that the vitreous body may promote disease in patients with diabetes. The findings of the present study could represent one mechanism that partially accounts for such an effect[26].

In conclusion, vitreous MPs reflect changes seen in progressive diabetic retinopathy including those seen with vitreous hemorrhage and proliferative diabetic retinopathy. The presence of blood in the vitreous of patients with diabetes may contribute to a diseased vitreous by evidence of increased Annexin-V^+ MPs, a sign of cellular apoptosis. Significant work is needed to further understand the role and function of MPs in the vitreous and how they may contribute to progression of diabetic eye disease.

Limitations Limitations to this study include the relatively small sample sizes in group comparisons. This study is also un-masked which may bias the results. Limitation in sample collection includes the variability in aspiration. Standardized aspiration rates have not been established as it is an assistant that collects the specimen in the 3 mL syringe. Despite establishing a vitrectomy cut-rate, person-to-person differences in aspiration rate can also change MP singlet populations which was not controlled in this study.

Most of the current analysis was done on vitreous specimens that were frozen. The current microparticle literature demonstrates that freezing MPs may alter MP integrity and artifactually increase the number of MPs seen by flow cytometry known as MP-fracture[27]. Due to the labor intensiveness of processing and evaluating samples the same day as sample collection, the majority of samples were still evaluated with a single freeze-thaw cycle, and thus the results should be interpreted with caution. A significant amount of clinical MP literature, however, supports a protocol where specimens have undergone a single freeze-thaw cycle.

The BD LSR II flow cytometer has a lower limit of detection at 0.488 μm, the wavelength of its forward scatter laser. This study only evaluates MPs that are between detectable range through 2.0 μm but not those that are smaller, in the 0.03 μm range. This study may bias its results towards MPs that are larger and neglecting smaller MPs that are beyond the lower detection-limit of the cytometer. Sample size calculations were not done to make the conclusions seen in the study and thus must be considered exploratory.

ACKNOWLEDGEMENTS

Foundation: Supported by an unrestricted grant to Washington University by Research to Prevent Blindness Inc., New York, NY.

Conflicts of Interest: Sultan H, None; **Rajagopal R,** None; **Rao PK,** None; **Piggott KD,** None; **Paley MA,** None; **Hassman LM,** None; **Li AS,** None; **Marshall B,** None; **Apte RS,** None.

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