• Basic Research •

Vascular endothelial growth factor/connective tissue growth factor and proteomic analysis of aqueous humor after intravitreal conbercept for proliferative diabetes retinopathy

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Abstract

● **AIM:** To investigate the role of connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) in the protein profile of the aqueous humor in patients with proliferative diabetic retinopathy (PDR) following intravitreal injection of conbercept.

● **METHODS:** This study included 72 PDR patients and 8 cataract patients as controls. PDR patients were divided into 3 groups according to the intervals of 3, 5, and 7d between intravitreal conbercept (IVC, 0.5 mg/0.05 mL) injection and pars plana vitrectomy (PPV) performed. Aqueous humor samples were collected before and after IVC and PPV for VEGF and CTGF levels detected with enzyme-linked immunosorbent assay (ELISA). The differential proteomics of 10 patients who underwent PPV surgery 5d after IVC and 8 normal controls was studied, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed on the data, and the protein interaction network of 23 differential proteins was studied.

● **RESULTS:** Post-IVC, VEGF levels decreased and CTGF levels increased significantly in aqueous humor, with the CTGF/VEGF ratio rising significantly at all intervals. Liquid chromatography tandem mass spectrometry (LC-MS/MS) identified differentially expressed proteins between pre- and post-IVC samples. GO and KEGG analyses revealed involvement in immune response, stress response, complement and coagulation cascades, ferroptosis, and PPAR signaling pathways. PPI analysis highlighted key proteins like APOA1, C3, and transferrin (TF). ELISA assay confirmed the differential expression of proteins such as HBA1, SERPINA1, COL1A1, and ACTB, with significant changes in the IVC groups.

● **CONCLUSION:** The study demonstrates that IVC effectively reduces VEGF levels while increasing CTGF levels, thereby modifying the CTGF/VEGF ratio, and IVC significantly alters the protein profile in the aqueous humor of patients with PDR. Proteomic analysis reveals that these changes are associated with critical biological pathways and protein interactions involved in immune response, stress response, and cellular metabolism.

● **KEYWORDS:** proliferative diabetic retinopathy; conbercept; vascular endothelial growth factor; connective tissue growth factor; proteomics

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INTRODUCTION

Diabetes retinopathy (DR) is the most common type of diabetes microvascular complications[1-2]. The main etiology is the damage of retinal microvessel caused by diabetes. According to the development stage and severity of DR, it is clinically divided into non proliferative diabetes retinopathy (NPDR) and proliferative diabetes retinopathy (PDR). PDR is a severe complication of diabetes mellitus (DM) and a leading cause of vision loss and blindness among working-age adults globally. Its pathogenesis involves complex interactions between metabolic control, genetic predisposition, and environmental factors[3]. Key pathological mechanisms include hyperactivity of the polyol pathway, activation of protein kinase C, accumulation of advanced glycation end products (AGEs), oxidative stress, and

overexpression of inflammatory cytokines. The prevalence of DR increases with the duration of diabetes and poor glycemic control. Approximately one-third of diabetic patients develop some form of retinopathy, with PDR representing the most advanced stage. As the global incidence of diabetes rises, the burden of DR, and specifically PDR, is expected to increase correspondingly.

PDR is characterized by the growth of new, fragile blood vessels on the surface of the retina in response to ischemia and hypoxia-induced vascular endothelial growth factor (VEGF) overexpression. These new vessels can bleed, leading to vitreous hemorrhage, and fibrous tissue proliferation, which can contract and cause tractional retinal detachment (TRD). These changes significantly threaten vision and can lead to complete blindness if untreated. The clinical features of NPDR include microaneurysm, retinal hemorrhage, intraretinal microvascular abnormalities and changes in vein diameter, while the characteristic marker of PDR is retinal neovascularization[4-5]. With the increase of the global incidence rate of diabetes, DR has become the main cause of adult visual impairment and blindness in developed countries[6].

At present, the treatment of diabetes ophthalmopathy mainly focuses on PDR, such as laser photocoagulation, intravitreal injection of anti-VEGF and steroids, vitreoretinal surgery, *etc*[7-8]. Laser photocoagulation has been the mainstay treatment for PDR for decades. It works by ablating ischemic retinal tissue, thereby reducing VEGF production and subsequent neovascularization. However, it can lead to loss of peripheral vision and night vision. Agents such as bevacizumab, ranibizumab, and aflibercept are used to inhibit VEGF activity, thereby reducing neovascularization and macular edema. Although effective, these treatments require frequent injections and monitoring. For advanced PDR with complications like vitreous hemorrhage or TRD, pars plana vitrectomy (PPV) is often required. This surgery removes the vitreous gel and associated fibrous tissue, relieving traction on the retina and clearing hemorrhage. The value of intravitreal conbercept (IVC) assisted PPV in PDR treatment has been widely recognized, which can shorten the operation time, reduce intraoperative bleeding, and make the operation simple and safe. However, while neovascularization atrophy is caused by the injection of anti-VEGF drugs, retinal fibrosis will also occur, and the degree will gradually increase, leading to an increased risk of TRD. If PPV operation is too early, the drug action time will be reduced and the drug effect will be affected due to the drug clearance effect of PPV operation. However, if the interval is too long, the proliferation membrane will shrink, and there is a risk of TRD[9-12]. Therefore, it is particularly important to choose the appropriate medication surgery interval. At present, there is no definite conclusion on the injection operation time interval of PPV assisted by preoperative anti-VEGF injection. Ali *et al*'s[13] study showed that for PDR patients, retinal fibrosis worsens 7d after the injection of anti-VEGF drugs, and the incidence of TRD 28d after the injection was as high as 71.43%. At present, the commonly used time interval is 3-5d. Ma *et al*'s[14] study showed that connective tissue growth factor (CTGF) was proportional to the degree of fibrosis, while VEGF was inversely proportional to the degree of fibrosis. The ratio of CTGF/VEGF was the strongest predictor of fibrosis. Therefore, the level of CTGF and the ratio of CTGF/VEGF will have important predictive value for the level of fibrosis. In this study, we selected patients with 3, 5, and 7d interval between injection and operation of conbercept. By measuring the changes of the concentration and ratio of VEGF/CTGF in aqueous humor before and after IVC, the degree of intraoperative fibrosis was observed to provide a theoretical basis for the drug injection surgery interval of IVC assisted PPV. However, due to side effects and individual differences, we urgently need new therapeutic methods to treat PDR. A better understanding of the pathological mechanism of this disease will help to explore new therapeutic methods. The research on PDR is very limited, because there is no disease model that can completely replicate the pathophysiological changes of the neurovascular system at all stages of the disease[8]. Because of the close anatomical and biological relationship between the retina and the vitreous body, the changes of retinal physiology and pathology can be reflected in the protein composition of the vitreous body, and the vitreous body can be easily obtained through routine surgery[15-17]. Because aqueous humor can reflect changes in vitreous composition, vitreous fluid obtained through vitrectomy can be used to indirectly analyze the physiological and pathological state of retina. Mass spectrometry has been widely used to study biomolecules, one of which is proteomics[18-19]. Age related macular disease[20-21], DR[22-23], and other eye diseases are of great significance. Although there have been some studies to analyze DR vitreous proteins[24-25], our understanding of the molecular pathology of this disease is far from enough. Quantitative proteomic analysis is an analytical chemical technique used to determine protein content, which is helpful to better explore the potential pathological mechanism of disease[26].

Here, we collected 72 PDR patients (72 eyes) and 8 cataract patients as normal controls. Enzyme-linked immunosorbent assay (ELISA) method was used to detect the effect of the interval between IVC and PPV (3, 5, 7d) on the concentration of VEGF and CTGF. In addition, 10 patients who underwent PPV surgery 5d after IVC and 8 normal controls were selected to analyze the changes of vitreous fluid protein level induced by IVC using tandem mass spectrometry proteomics method.

We also analyzed the distribution of proteins and made bioinformatics analysis, including protein function, related diseases, gene ontology (GO), protein protein interaction (PPI), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Our study highlights the importance of considering patient heterogeneity in clinical research. By correlating changes in protein expression with clinical parameters, we can gain a deeper understanding of the molecular mechanisms and clinical impacts of IVC treatment in PDR patients. This approach helps optimize treatment strategies and provides a foundation for personalized therapies, thereby improving efficacy and reducing complications.

SUBJECTS AND METHODS

Ethical Approval This study was a prospective, clinical case-control study, which was approved by the Ethics Committee of Tongji Hospital. All participants signed the informed consent form before joining the group.

Source of Cases Totally 72 PDR patients (72 eyes) treated in Shanghai New Vision Eye Hospital from June 2018 to December 2020 were collected, including 42 female patients (42 eyes), 30 male patients (30 eyes) and 8 cataract patients as normal controls. All patients received general ophthalmic examination after admission, such as best corrected visual acuity (BCVA), intraocular pressure (IOP), fundus examination after mydriasis under slit lamp, optical coherence tomography (OCT) and/or ocular B-ultrasound examination.

Inclusion Criteria of Participants The inclusion criteria of participants were as follows: aged 18-70; gender is not limited; vitreous hemorrhage and/or retinal neovascularization (RNV); those who need and are willing to accept the injection of conbercept, phacoemulsification and/or vitrectomy; detailed eye examination of all subjects was in line with PDR; the patient's blood sugar was stable.

Exclusion Criteria for Participants The exclusion criteria for participants were as follows: merging other eye diseases that may cause vision loss, such as retinal detachment; History of vitrectomy; history of intraocular injection of anti-VEGF, triamcinolone acetonide (TA), *etc*., and retinal laser within 6mo before admission; Merged neovascular glaucoma (NVG); severe eye infection; using anticoagulant or antiplatelet drugs; poor control of diabetes before or after operation; serious systemic diseases; pregnant women, bipolar disorder, schizophrenia and other serious mental diseases; allergy to conbercept.

Withdrawal Criteria for Participants The withdrawal criteria for participants were as follows: other retinal diseases (such as rhegmatogenous retinal detachment) were found during vitrectomy. Vitreous hemorrhage caused by other reasons; Participants are unwilling to continue to participate; The patient voluntarily withdrew from the clinical investigator.

Research Group All PDR patients in this study were randomly divided into three groups: patients received intravitreal injection of conbercept (0.5 mg/0.05 mL, Chengdu Kanghong Biotechnology Co., Ltd., China) 3 to 7d before PPV operation, anterior chamber puncture before injection, 0.05 mL aqueous humor was extracted, and 0.05 mL aqueous humor was extracted again before PPV operation. The specific groups were as follows: Group A (control group): 8 cases (8 eyes) of cataract patients were taken aqueous humor during cataract surgery as the control. Group B (IVC 3d group): The patient received IVC injection 3d before PPV operation, aqueous humor was taken before injection, and again before PPV operation. Group C (5d group): patients received IVC injection 5d before operation, aqueous humor was taken before injection, and again before PPV operation. Group D (7d group): Patients received IVC injection 7d before operation, aqueous humor was taken before injection and again before PPV operation.

Routine Inspection International standard logarithmic visual acuity chart was used for BCVA examination. To facilitate statistics, the measured visual acuity is converted to logMAR with the conversion formula of logMAR=lg(1/decimal visual acuity). IOP measure with non-contact tonometer, and record the average value of three consecutive measurements. After mydriasis, the fundus examination was performed with a prescope under a slit lamp. The retina was examined by OCT or B ultrasound.

Intravitreal Conbercept Injection Before the operation, the patient first used levofloxacin eye drops. The injection was carried out in the operating room. The patient lay flat on the operating bed, anesthetized the eyes with alcaine, disinfected the eyes, and extracted 0.05 mL of conbercept. The patient was instructed to look at the nose. The needle was inserted at an angle of 3.5-4 mm behind the temporal corneal margin of the patient, penetrating the sclera. The direction of the needle was at the center of the eye. After the observation of the pupil, it was found that the position of the needle was correct, 0.05 mL of conbercept was injected into the vitreous cavity, the needle was pulled out, and the puncture port was pressed for 5-10s. The patient's vision was checked and determined manually. Ofloxacin eye ointment (Xingqi, Shenyang, China) was applied in the conjunctival sac, and sterile gauze was applied to the surgical eye cover. The operation is performed by the same clinician, who has rich experience. The obtained aqueous humor was stored at - 80°C.

BCVA, IOP, anterior and posterior segment reaction of eyeball, and fundus examination with slit lamp were examined the second day after operation.

Vitrectomy Aqueous Humor Extraction PPV operation was performed 3-7d after IVC. The operation was performed

by the same retinal doctor with rich experience. The patient had sufficient mydriasis before the operation. For example, the mixed solution of lidocaine and bupivacaine was used to conduct retrobulbar and peribulbar nerves, 1 mL sterile syringe with 30G needle was used to inject into the limbus of the cornea to obtain about 0.05 mL aqueous humor, and then standard 25G three channel minimally invasive vitrectomy was performed. The obtained aqueous humor was stored at -80°C. Vitrectomy was performed with Constellation (Alcon, USA) and Resight 500 (Carl Zeiss Meditec, USA) at a cutting rate of 5000/min. Use intraocular electrocoagulation and laser as needed to peel off the fibrous vascular membrane. Carry out panretinal photocoagulation (PRP), and the laser shall at least cross the equator. Fill the vitreous cavity with air, perfusion fluid or silicone oil. The fibrovascular membrane proliferation was graded according to the following criteria during operation: Grade 0: no adhesion; Grade 1: focal adhesions only; Grade 2: extensive adhesion >1 site or in the optic papilla, macula and vascular arch; Grade 3: Adhesion extends to the periphery of the retina. Tobramycin and dexamethasone eye drops were given to the eyes after the operation, and the complications and vitreoretinal adhesions were recorded.

Aqueous Humor Extraction During Phacoemulsification The patient was placed in a supine position, and underwent corneal surface anesthesia. A 2.2 mm incision was made at the limbus of the cornea. About 0.05 mL aqueous humor was extracted with a 30G injection needle, and then conventional cataract phacoemulsification and intraocular lens implantation were performed.

Enzyme-Linked Immunosorbent Assay In this study, the concentration of VEGF and CTGF is detected by ELISA according to the results as follows: Take out the preserved aqueous humor sample, 1000×G centrifuge for 20min, take the supernatant for detection, set the standard hole and sample hole, add 50 μL standard of different concentrations to the standard quality control, and add 50 μL sample to the sample hole. Add 100 μL of HRP labeled test antibody to the standard hole and sample hole, seal the reaction hole with a sealing membrane, incubate it in a 37°C incubator for 60min, discard the liquid, pat dry on the absorbent paper, fill each hole with detergent, stand for 1min, shake off the detergent, pat dry on the absorbent paper, and wash the plate repeatedly for 5 times. Add 50 μL of substrate A and B into each well. Within 15min, measure the optical density (OD) value of each well at the wavelength of 450 nm. With the measured OD value of the standard as the abscissa and the concentration value of the standard as the ordinate, draw a standard curve and obtain a linear regression equation. Substitute the OD value of the sample into the equation to calculate the concentration of the sample.

Proteomics Analysis According to the concentration of VEGF and CTGF in aqueous humor of each group, we randomly selected aqueous humor samples from 10 cases IVC 5d group and 8 control group for proteomic study, and mixed 10 samples from each PDR group and 8 samples from each IVC group. Agilent MARS-14 high-capacity affinity column (4.6×100 mm) was used to remove 14 most abundant proteins (serum albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and thyrotropin). Subsequently, the peptide segment was digested by 20 ng/μL trypsin protease for 16-18h, and 100 μg samples were taken from each group. Peptides after digestion, samples were analyzed with LC-MS/MS instrument, the mass range was set as 350-1800 m/z, and the resolution was 70 000 m/z. Automatic gain control target is set to 3×10^6, the maximum injection time is set as 30ms.

Verification of Differential Protein Expression by ELISA ELISA kit was used to detect the concentration of HBA1, SERPINA1, COL1A1, ACTB, CLU, APOE, AFM, ALB, TF, PLG, GC, APOA4, LTBP2, EFEMP1, CFI, CFH, C4B, C3, FGA and other proteins in the vitreous, and complete the experiment according to the experimental instructions. Calculate the protein concentration according to the OD value and dilution ratio.

Statistical Analysis The differentially expressed proteins were analyzed by R language edgeR package. Set Benjamin Hochberg error detection rate (FDR) 1.5. Utilize XGR software (http://galahad.well.ox.ac.uk:3040) GO and KEGG of differentially expressed proteins were analyzed. PPI was from STRING Database (https://STRING-db.org, 11.0). The age of patients and onset time of DM in group B, C, and D were tested by normality test, which were consistent with the normal distribution. Analysis of variance was used. Sex is a categorical variable, and R×C test is used. SPSS 23.0 statistical analysis software was used for statistical analysis. The results of measurement data were represented by mean±standard derivation. After normal distribution and homogeneity of variance tests, if the data meet the normality, one-way analysis of variance was conducted. SNK-Q test was used for pairwise comparison between two groups, and median was used for measurement data of non-normal distribution. The *t*-test was used for comparison between two groups. The difference was statistically significant with $P<0.05$.

RESULTS

General Information of the Patient There were 8 cases (8 eyes) in control group, 5 males (5 eyes) and 3 females (3 eyes). The age was 57.7±9.7 years old. The general information of the PDR patients was showed in the Table 1. There is no significant difference between the two groups in terms of

Table 1 General information of patients

Subject	Group B	Group C	Group D	*P*
Number of cases	24	24	24	
Sex				0.843
Male	10	9	11	
Female	14	15	13	
Age	58.9±11.1	56.8±13.2	57.7±9.7	0.121
Eyes				0.722
Right eye	17	15	16	
Left eye	7	10	8	
Cataract surgery				0.926
Normal lens	18	19	18	
Intraocular lens	6	5	6	
Course of diabetes (y)	23.3±12.7	21.1±10.4	22.2±12.1	0.102
Preoperative intraocular pressure	14.6±4.4	15.2±3.1	14.9±2.6	0.2762
Preoperative BCVA (logMAR)	1.71±0.23	1.82±0.15	1.77±0.12	0.120
Surgical indications				0.393
Fibrovascular membrane	3	4	1	
Vitreous hemorrhage	9	8	11	
TRD	2	1	4	
Merge 2 or more	10	11	8	

BCVA: Best corrected visual acuity; TRD: Tractional retinal detachment.

Table 2 Evaluation of intraoperative vitreoretinal adhesions and complications

Intraoperative evaluation	Group B (IVC 3d)	Group C (IVC 5d)	Group D (IVC 7d)	*P*
Intraoperative complications				0.922
Iatrogenic retinal tear	4 (16.7%)	3 (12.5%)	5 (20.8%)	
Intraocular tamponade				0.883
Air	11 (45.8%)	9 (37.5%)	9 (37.5%)	
Perfusate	9 (37.5%)	8 (33.3%)	11 (45.8%)	
Silicone oil	4 (16.7%)	6 (25.0%)	4 (16.7%)	
Classification of adhesion degree of proliferating membrane				0.727
Level 0	2	3	1	
Level 1	3	4	6	
Level 2	11	10	13	
Level 3	8	7	4	

IVC: Intravitreal conbercept.

gender, onset time of DM, preoperative BCVA, and surgical indications, which is comparable.

Intraoperative Vitreoretinal Adhesions and Complications There were 4 cases (16.7%) in group B, 3 cases (12.5%) in group C, and 5 cases (20.8%) in group D, respectively occurred retinal tear (P=0.922). In terms of tamponade, there was no statistical difference between the three groups (P=0.883), and there was no statistical difference between the three groups (P=0.727) in terms of the adhesion degree of the proliferating membrane (Table 2).

Concentration Changes of VEGF and CTGF Before and After Injection of Conbercept ELISA was used to test the concentrations of VEGF and CTGF, as showed in the Figure 1A, the concentrations of VEGF and CTGF in aqueous humor of patients with PDR were 178.61±27.21 pg/mL and 765.43±53.99 pg/mL respectively, while the concentrations of VEGF and CTGF in aqueous humor of patients without PDR were 125.35±13.04 pg/mL and 324.33±82.75 pg/mL, respectively. The concentrations of VEGF and CTGF in aqueous humor of PDR patients were higher than those of normal control group (P<0.05). After IVC of PDR patients, the concentration of VEGF in aqueous humor showed a downward trend and that of CTGF showed an upward trend. The concentrations at 3, 5 and 7d were statistically different (Figure 1B). Figure 1C showed ratio of CTGF/VEGF in aqueous humor after injection of conbercept, after IVC, the ratio of CTGF/VEGF in aqueous humor increased gradually, and the ratio of CTGF and VEGF in each group was statistically significant.

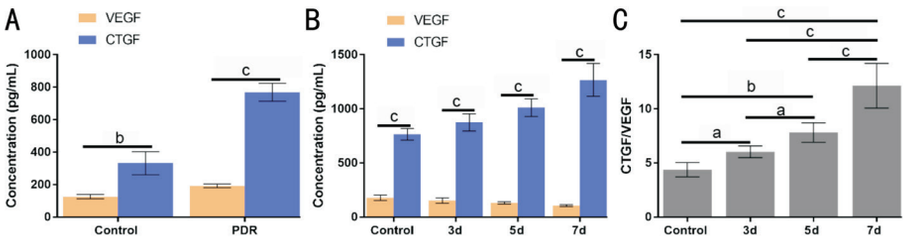

Figure 1 Concentration changes of VEGF and CTGF before and after injection of conbercept A: VEGF and CTGF in aqueous humor of patients with or without PDR; B: The concentrations of VEGF and CTGF in aqueous humor of PDR patients before and after IVC conbercept for 3, 5, 7d; C: The ratio of CTGF/VEGF in aqueous humor after injection of conbercept. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$.

Table 3 Identification by mass spectrometry and screening of the differentially expressed proteins identificated by MS of IVC 5d group or IVC 7d group compared to the patients before operation *n*

Parameters	Down regulated proteins	Up regulated proteins	Differentially expressed proteins
IVC 5d *vs* before operation	21	18	39
IVC 7d *vs* before operation	24	26	50

IVC: Intravitreal conbercept.

Differentially Expressed Proteins Between Before and After IVC for 5d or 7d Differential expression proteins were screened according to the standard that the multiple change was more than 2.0 times (up regulation was more than 2.0 times or down regulation was less than 0.5 times) and *P*-value was less than 0.05. IVC 5d *vs* before operation, a total of 39 differential expression proteins were detected, of which 21 proteins were down regulated and 18 proteins were up-regulated. IVC 7d *vs* before operation, a total of 50 differential expression proteins were detected, of which 24 proteins were down regulated and 26 proteins were up-regulated (Table 3).

Function enrichment analysis of differentially expressed proteins

1) Gene ontology In proteomics research, it is necessary to summarize and analyze the studied proteins and their functions from a more systematic and general level and perspective. In this project, the differential expression proteins of before operation and after IVC for 5d or 7d, each comparison group were analyzed with GO enrichment by Fisher exact test. Classified according to biological processes (BP), differentially expressed proteins in IVC 5d group vitreous participated in immunoglobulin mediated immune response (42%), response to stress (8%), defense response (8%), localization (8%), endocytosis (6%) *etc*. (Figure 2A). Differentially expressed proteins in IVC 7d group vitreous participated in immune effector process (33%), platelet degranulation (26%), transport (18%), vesicle-mediated transport (5%), response to wounding (5%) *etc*. (Figure 2B). Classification of cell components (CC) showed that differentially expressed proteins in IVC 5d group involve in extracellular region part (86%), membrane-bounded organelle (8%), plasma membrane (3%), membrane-bounded vesicle (3%) *etc*. (Figure 2C). Differentially expressed proteins in IVC 7d group involved in extracellular membrane-bounded organelle (85%), vesicle (10%), membrane-bounded organelle (3%), extracellular space (3%) *etc*. (Figure 2D). The analysis of its molecular function (MF) showed that differentially expressed proteins in IVC 5d group involved in protein binding (53%), antigen binding (36%), binding (6%), extracellular matrix structural constituent (3%), enzyme inhibitor activity (3%) *etc*. (Figure 2E). Differentially expressed proteins in IVC 7d group mainly involved in protein binding (97%) *etc*. (Figure 2F).

2) KEGG pathway significant enrichment analysis KEGG was applied to obtain the analysis results. According to the results, differentially expressed proteins in IVC 5d group involved in complement and coagulation cascades (6%), African trypanosomiasis (6%) *etc*. (Figure 3A). differentially expressed proteins in IVC 7d group involved in complement and coagulation cascades (26%), African trypanosomiasis (8%), arachidonic acid metabolism (5%), ferroptosis (5%), PPAR signaling pathway (3%), pertussis (3%), thyroid hormone synthesis (3%), cholesterol metabolism (3%) *etc*. (Figure 3B).

3) Protein-protein interaction analysis By use of string online data base, protein-protein interaction (PPI) analysis was performed. APOA2, TIMP1, ITIH2, APCS, SERPINA4 *etc*. involved in the PPI net of differentially expressed proteins in IVC 5d group (Figure 4A). In the IVC 7d group, the PPI net of differentially expressed proteins was more complex and results was showed in the Figure 4B, APOA1, C3, TF, TTR, AHSG, FGA, PLG, Serpina1, CST3, RBP4, AZGP1, AACT *etc*. was involved in the PPI network.

Differentially Expressed Proteins After IVC for 5d or 7d Differential expression proteins were screened according to the standard that the multiple change was more than 2.0 times (up regulation was more than 2.0 times or down regulation was less than 0.5 times) and *P*-value was less than 0.05. Among

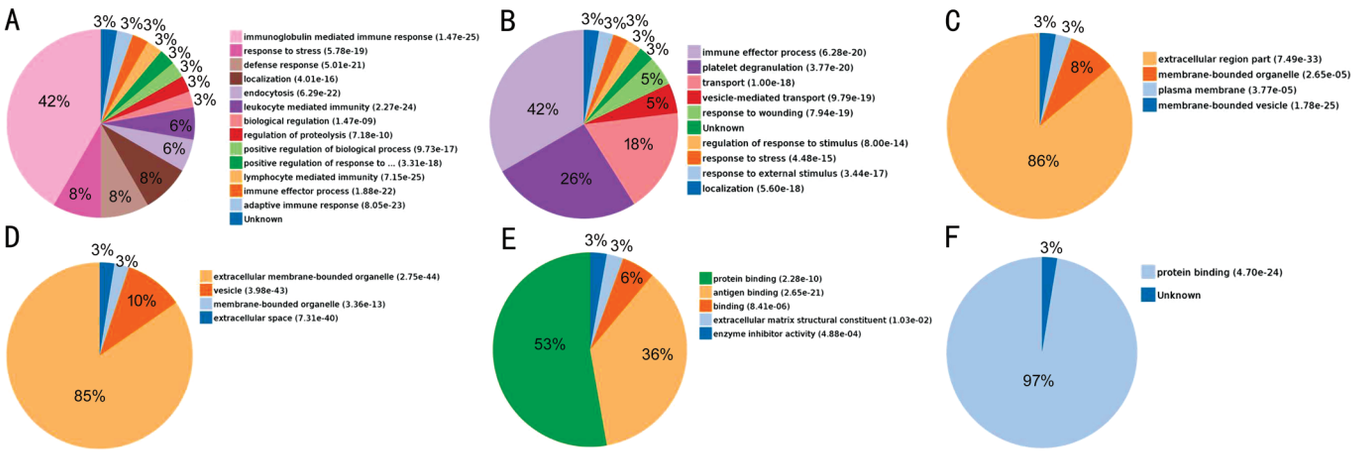

Figure 2 Gene Ontology (GO) analysis of differentially expressed proteins between before operation and after IVC for 5d or 7d A, B: Biological processes (BP) analysis of differentially expressed proteins in IVC 5d or 7d group vitreous participated. C, D: Classification of cell components (CC) of differentially expressed proteins in IVC 5d or 7d group; E, F: The analysis of molecular function (MF) of differentially expressed proteins in IVC 5d or 7d group. IVC: Intravitreal conbercept.

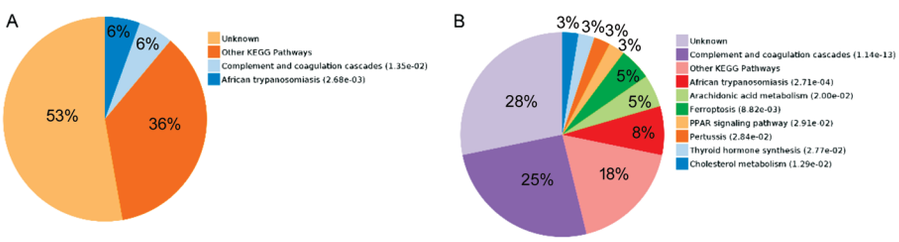

Figure 3 KEGG analysis of differentially expressed proteins between before operation and after IVC for 5d or 7d A: KEGG analysis of differentially expressed proteins in IVC 5d; B: KEGG analysis of differentially expressed proteins in IVC 7d group. IVC: Intravitreal conbercept; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 4 Identification by mass spectrometry and screening of the differentially expressed proteins identified by MS of IVC 5d group or IVC 7d group compared to the cataract control group

Parameters	Down regulated proteins	Up regulated proteins	Differentially expressed proteins
IVC 5d *vs* control	22	6	28
IVC 7d *vs* control	16	5	21

IVC: Intravitreal conbercept.

IVC 5d *vs* control, a total of 28 differential expression proteins were detected, of which 22 proteins were down regulated and 6 proteins were up-regulated. Among IVC 7d *vs* before operation, a total of 21 differential expression proteins were detected, of which 16 proteins were down regulated and 5 proteins were up-regulated (Table 4).

Function enrichment analysis of differentially expressed proteins

1) Gene ontology In proteomics research, it is necessary to summarize and analyze the studied proteins and their functions from a more systematic and general level and perspective. In this project, the differential expression proteins of each comparison group were analyzed with GO enrichment by Fisher exact test: Classified according to BP, differentially expressed proteins in IVC 5d group vitreous participated in response to wounding (64%), response to external stimulus (8%), vesicle-mediated transport (8%), response to stimulus (4%), transport (4%), multi-organism process (4%), response to stress (4%), acute inflammatory response (4%) *etc*. (Figure 5A). Classified according to BP, differentially expressed proteins in IVC 7d group vitreous participated in response to wounding (75%), localization (5%), multi-organism process (5%), response to external stimulus (5%), positive regulation of response to stimulus (5%), protein activation cascade (5%; Figure 5B). Classification of CC showed that differentially expressed proteins in IVC 5d group involved in blood microparticle (64%), extracellular space (32%), membrane-bounded organelle (4%) *etc*. (Figure 5C). Differentially expressed proteins in IVC 7d group involved in extracellular membrane-bounded organelle (95%) and extracellular space (5%; Figure 5D). The analysis of its MF showed that differentially expressed proteins in IVC 5d group involved in protein binding (92%), serine-type endopeptidase activity (4%) *etc*. (Figure 5E). Differentially expressed proteins in IVC 7d

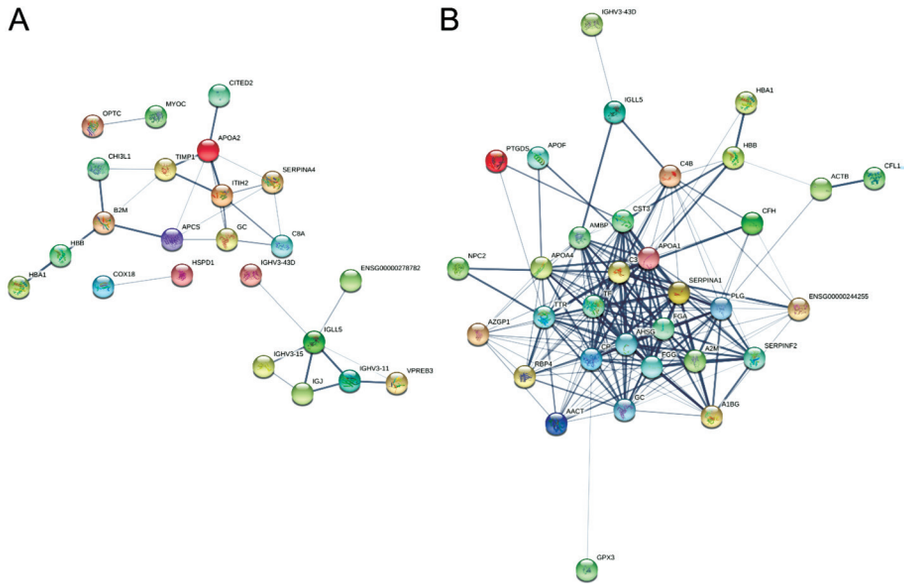

Figure 4 Protein-protein interaction analysis of differentially expressed proteins between before operation and after IVC for 5d or 7d A: Protein-protein interaction analysis of differentially expressed proteins in IVC 5d; B: Protein-protein interaction analysis of differentially expressed proteins in IVC 7d group. IVC: Intravitreal conbercept.

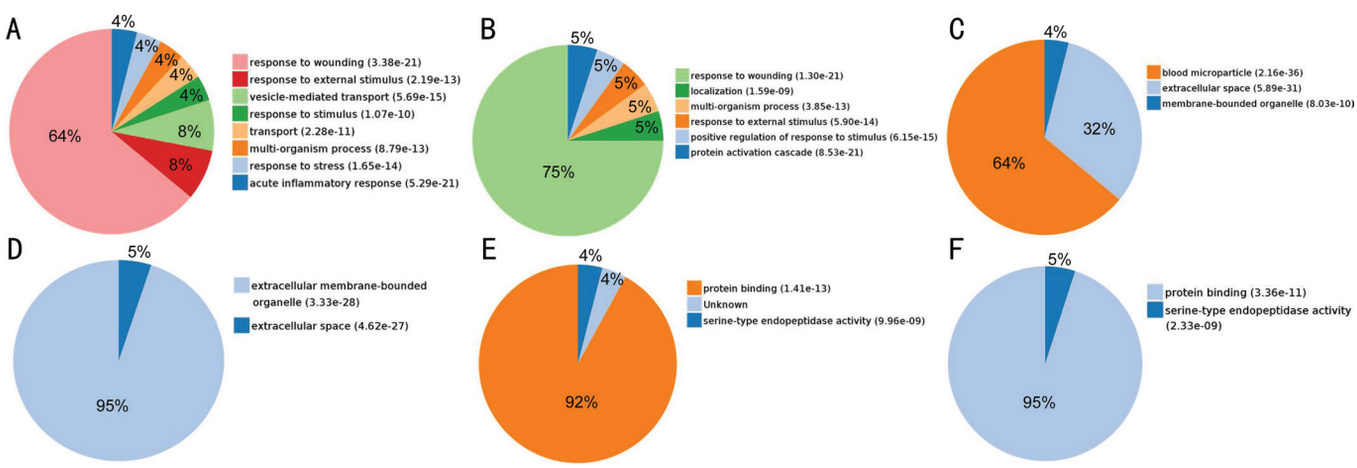

Figure 5 Gene ontology (GO) analysis of differentially expressed proteins between cataract control group and after IVC for 5d or 7d A, B: Biological processes (BP) analysis of differentially expressed proteins in IVC 5d or 7d group vitreous participated compared with cataract control group; C, D: Classification of cell components (CC) of differentially expressed proteins in IVC 5d or 7d group compared with cataract control group; E, F: The analysis of molecular function (MF) of differentially expressed proteins in IVC 5d or 7d group compared with cataract control group. IVC: Intravitreal conbercept.

group mainly involved in protein binding (95%) and serine-type endopeptidase activity (5%; Figure 5F).

2) KEGG pathway significant enrichment analysis According to the results, differentially expressed proteins in IVC 5d group involved in complement and coagulation cascades (36%), relaxing signaling pathway (4%), platelet activation (4%) *etc*. (Figure 6A). Differentially expressed proteins in IVC 7d group involved in complement and coagulation cascades (45%), Cholesterol metabolism (10%) *etc*. (Figure 6B).

3) Protein-protein interaction analysis By use of string online data base, PPI analysis was performed. Totally 23 proteins were involved in the PPI net of differentially expressed proteins in IVC 5d group (Figure 7A). In the IVC 7d group, totally 18 proteins were involved in PPI net of differentially expressed proteins (Figure 7B).

DISCUSSION

At present, the research on the pathogenesis of DR primarily includes the hyperactivity of the polyol pathway, activation of protein kinase C, accumulation of AGE, oxidative stress, and overexpression of cytokines[27]. Extensive retinal ischemia and hypoxia cause blood-retinal barrier damage, induce vascular leakage and macular edema, and lead to the proliferation of fibrovascular membranes at the vitreoretinal interface, causing vitreous hemorrhage and retinal detachment, which seriously threaten the patient's vision[28-30]. These pathological processes involve the interaction of multiple proteins[31]. Inflammatory

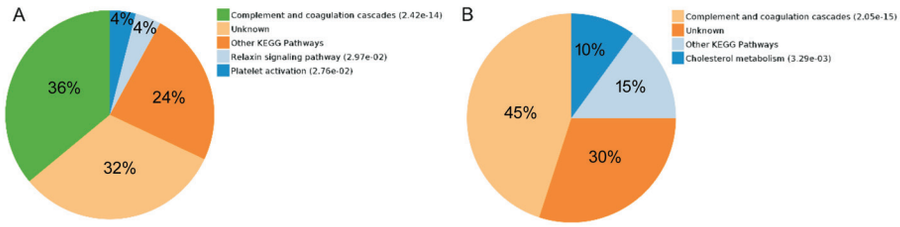

Figure 6 KEGG analysis of differentially expressed proteins between cataract control group and after IVC for 5d or after IVC for 7d A: KEGG analysis of differentially expressed proteins in in IVC 5d group compared with cataract control group; B: KEGG analysis of differentially expressed proteins in IVC 7d group compared with cataract control group.

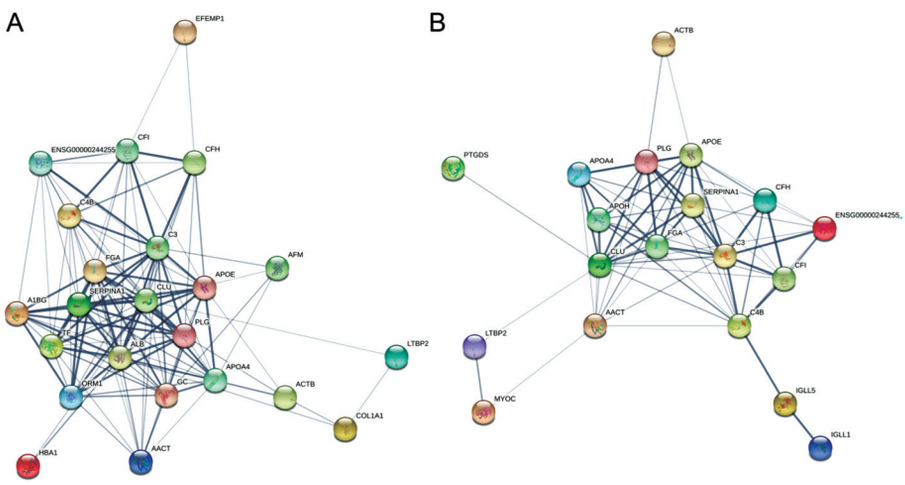

Figure 7 Protein-Protein interaction analysis of differentially expressed proteins between cataract control group and after IVC for 5d or after IVC for 7d A: Protein-Protein interaction analysis of differentially expressed proteins in in IVC 5d group compared with cataract control group; B: Protein-Protein interaction analysis of differentially expressed proteins in IVC 7d group compared with cataract control group.

and angiogenic factors, especially VEGF, are key to retinal neovascularization. Although VEGF antagonists have proven effective in treating PDR, their efficacy is short-lived and may aggravate fibrosis and cause traction retinal detachment. To further explore the role of VEGF antagonists in treating PDR, we administered conbercept to PDR patients 3, 5, and 7d before PPV. Aqueous humor samples were collected before and after injection. The results showed a downward trend in VEGF levels and an upward trend in CTGF levels post-IVC. The ratios of CTGF/VEGF increased significantly across all time points, indicating a therapeutic impact on angiogenesis and fibrosis pathways in PDR[32]. To comprehensively measure the impact of IVC on PDR vitreous protein levels, analyze the pathogenesis of PDR, and explore new therapeutic targets, we employed proteomics. The results showed that differentially expressed proteins in the 5d IVC group were involved in immunoglobulin-mediated immune responses (42%), stress responses (8%), defense responses (8%), localization (8%), and endocytosis (6%). The 7d IVC group was involved in immune response processes (33%), platelet degranulation (26%), transport (18%), and wound response (5%). In CC analysis, differentially expressed proteins in the 5d IVC group mainly involved extracellular regions (86%) and membrane-bound organelles (8%). The 7d IVC group mainly involved extracellular membrane-bound organelles (85%) and vesicles (10%). In MF analysis, differentially expressed proteins in the 5d IVC group mainly participated in protein binding (53%) and antigen binding (36%); the 7d IVC group mainly participated in protein binding (97%). KEGG analysis showed that differentially expressed proteins in the 5d IVC group were involved in complement and coagulation cascade reactions (6%); the 7d IVC group involved complement and coagulation cascade reactions (26%), arachidonic acid metabolism (5%), and ferroptosis (5%). PPI analysis showed that APOA2, Timp1itih2, and APCS in the 5d IVC group, and APOA1, C3, and TF in the 7d IVC group, participated in the PPI network. ELISA results validated these findings, showing high expression of HBA1, SERPINA1, and COL1A1, and low expression of CLU, APOE, and ALB.

Here, we analysed the vitreous humor protein profiles in PDR patients following IVC treatment reveals significant insights into the pathogenesis and potential therapeutic targets for this condition. However, it is crucial to acknowledge the potential heterogeneity within the PDR patient group. This variability may stem from differences in disease severity, duration of diabetes, genetic factors, and comorbid conditions, all of which can influence the protein expression profiles and treatment responses. To manage this variability and ensure

robust and meaningful results, our study included several strategies. First, we stratified patients into subgroups based on the interval between IVC and PPV surgery (3, 5, and 7d). This stratification helps account for differences in drug response times and the dynamic changes in protein expression post-treatment. Additionally, we employed strict inclusion and exclusion criteria to minimize confounding factors. For instance, patients with other significant eye diseases, recent vitrectomy, severe infections, or poorly controlled diabetes were excluded from the study. Further, we performed comprehensive proteomic analyses, including GO, KEGG, and PPI, to identify differentially expressed proteins and their involvement in various BP and pathways. By comparing the protein profiles of PDR patients before and after IVC treatment, and against cataract controls, we could pinpoint specific proteins and pathways that are altered due to the treatment. This approach not only highlights the therapeutic effects of IVC but also elucidates the underlying molecular mechanisms. Moreover, to validate our findings, we used ELISA to confirm the differential expression of key proteins identified through mass spectrometry. This dual-method validation strengthens the reliability of our results. Despite these measures, we recognize that individual variations in protein expression and disease progression can still impact the study outcomes. Therefore, future research should consider larger sample sizes and more diverse patient populations to further validate and refine these findings. In summary, in addition to CTGF and VEGF, Conbercept can affect the levels of various proteins in the vitreous of PDR patients, and the changes of differentially expressed proteins are also the key way to cause the aggravation of preretinal proliferative membrane. our study underscores the importance of considering patient heterogeneity in clinical research. Through careful stratification, rigorous selection criteria, and multi-faceted analytical approaches, we can manage this variability and derive meaningful insights into the effects of IVC on PDR, paving the way for more targeted and effective therapies. When analyzing the vitreous protein mass spectrometry of PDR patients after IVC treatment, some differentially expressed proteins identified were significantly correlated with clinical parameters such as vision and retinal thickness. By combining these clinical results with protein data, we not only deepened the interpretation of the research results, but also improved their practical significance. For example, we found that patients with significantly reduced VEGF levels also showed significant improvement in visual acuity and reduced retinal thickness. This indicates that IVC treatment not only plays a role at the molecular level, but also brings practical benefits in clinical practice. In addition, the increase in CTGF levels is associated with improved surgical outcomes, such as reduced intraoperative bleeding and increased visibility during PPV surgery. Specifically, in the IVC 5d and 7d groups, the expression changes of key proteins such as APOA1, C3, and TF are closely related to clinical outcomes. The involvement of these proteins in the PPI network indicates their important role in regulating immune responses, stress responses, and cellular metabolic processes. The high expression of APOA1 is associated with significant improvement in vision, indicating that this protein may play an important role in retinal repair and functional recovery. The high expression of C3 and TF is associated with structural improvement of the retina, particularly in reducing inflammation and promoting retinal stability. In addition, the high expression of proteins such as HBA1, SERPINA1, and COL1A1 is also closely related to the improvement of vision in patients. As a part of hemoglobin, the increased expression of HBA1 may improve the oxygenation status of local tissues, thereby contributing to the functional recovery of the retina. SERPINA1, as an anti-protease, may promote visual recovery by reducing inflammation and tissue damage. COL1A1, as a type of collagen, its high expression may play an important role in the structural repair of the retina. On the contrary, the low expression of proteins such as CLU, APOE, and ALB is associated with a decrease in retinal thickness. The low expression of CLU may reduce the occurrence of cell apoptosis, thereby protecting retinal cells. The low expression of APOE may be related to the improvement of lipid metabolism and the protection of retinal cells. The low expression of ALB may reduce protein aggregation in the retina, thereby reducing tissue damage and inflammatory response[33-37].

This study has limitations, including a sample size that may not fully capture the variability within the PDR patient population. Larger, more diverse cohorts are needed to validate findings. While we focused on proteomic changes at specific intervals post-IVC, longitudinal studies are necessary to understand long-term effects. The complexity of PDR suggests that other unidentified proteins and pathways might be involved, necessitating advanced proteomic techniques and integrative approaches. Future research should expand to larger, diverse cohorts, conduct long-term follow-ups, and use integrative omics approaches. Investigating the roles of key proteins like APOA1, C3, and TF in retinal repair, and exploring personalized treatment strategies based on proteomic profiles, will optimize therapeutic efficacy and minimize adverse effects.

In short, our study underscores the importance of considering patient heterogeneity in clinical research. By correlating protein expression changes with clinical parameters, we better understand the molecular mechanisms of IVC treatment in PDR patients and its clinical impacts. This helps optimize

treatment strategies and provides a basis for personalized treatments, improving efficacy and reducing complications. Future research should continue to explore these associations in larger, diverse cohorts to validate and expand our findings. Our work lays the foundation for exploring new therapeutic targets for PDR.

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