• Basic Research •

Characterization and validation of a chronic retinal neovascularization rabbit model by evaluating the efficacy of anti-angiogenic and anti-inflammatory drugs

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Abstract

● **AIM:** To establish a rabbit model with chronic condition of retinal neovascularization (RNV) induced by intravitreal (IVT) injection of DL-2-aminoadipic acid (DL-AAA), a retinal glial (Müller) cell toxin, extensive characterization of DL-AAA induced angiographic features and the suitability of the model to evaluate anti-angiogenic and anti-inflammatory therapies for ocular vascular diseases.

● **METHODS:** DL-AAA (80 mmol/L) was administered IVT into both eyes of Dutch Belted rabbit. Post DL-AAA delivery, clinical ophthalmic examinations were performed weekly following modified McDonald-Shadduck Scoring System. Color fundus photography, fluorescein angiography (FA), and optical coherence tomography (OCT) procedures were performed every 2 or 4wk until stable retinal vascular leakage was observed. Once stable retinal leakage (12wk post DL-AAA administration) was established, anti-vascular endothelial growth factor (VEGF) (bevacizumab, ranibizumab and aflibercept) and anti-inflammatory (triamcinolone, TAA) drugs were tested for their efficacy after IVT administration. Fluorescein angiograms were scored before and after treatment following a novel grading system, developed for the DL-AAA rabbit model.

● **RESULTS:** Post DL-AAA administration, eyes were presented with moderate to severe retinal/choroidal inflammation which was accompanied by intense vitreous flare and presence of inflammatory cells in the vitreous humor. Retinal hemorrhage was restricted to the tips of neo-retinal vessels. FA revealed maximum retinal vascular leakage at 2wk after DL-AAA injection and then persisted as evidenced by stable mean FA scores in weeks 8 and 12. Retinal vascular angiographic and tomographic features were stable and consistent up to 36mo among two different staggers induced for RNV at two different occasions. Day 7, mean FA scores showed that 1 μg/eye of bevacizumab, ranibizumab, aflibercept and 2 μg/eye of TAA suppress 65%, 90%, 100% and 50% retinal vascular leakage, respectively. Day 30, bevacizumab and TAA continued to show 66% and 44% suppression while ranibizumab effect was becoming less effective (68%). In contrast, aflibercept was still able to fully (100%) suppress vascular leakage on day 30. On day 60, bevacizumab, ranibizumab and TAA showed suppression of 7%, 12%, and 9% retinal vascular leakage, respectively, however, aflibercept continued to be more effective showing 50% suppression of vascular leakage.

● **CONCLUSION:** The DL-AAA rabbit model mimics RNV angiographic features like RNV and chronic retinal leakage. Based on these features the DL-AAA rabbit model provides an invaluable tool that could be used to test the therapeutic efficacy and duration of action of novel anti-angiogenic formulations, alone or in combination with anti-inflammatory compounds.

● **KEYWORDS:** DL-2-aminoadipic acid; chronic wet age-related macular degeneration; retinal neovascularization; animal model; anti-vascular endothelial growth factor drugs; fluorescein angiography

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INTRODUCTION

Angiogenesis is a complex physiological process, important for both vascular development and pathological condition like neovascularization. Dysregulated cascade of angiogenesis in the eye causes pathologies in retinal/choroidal vascular bed that may lead to partial or

complete vision loss, such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), neovascular age-related macular degeneration (nAMD), neovascular glaucoma, and corneal neovascularization[1-3]. Ocular vascular pathological conditions are characterized by increased vascular permeability and growth of new vessels which may bleed or leak exudates and caused retinal edema followed by fibrous scar that destroys the photoreceptor cells in the retina[4]. Human and animals studied revealed that vascular endothelial growth factor (VEGF) is a key pathogenic factor for aforementioned ocular vascular diseases, therefore, intravitreal (IVT) administration of anti-VEGF therapies have become the most common treatment modality for many retinal diseases[5-7]. In past two decades, anti-VEGF therapies have had tremendous success and revolutionized the treatment for ocular vascular diseases. Nonetheless, current anti-VEGF (bevacizumab, ranibizumab and aflibercept) therapies have several limitations within clinical use, namely 1) short duration effect, 2) repeated IVT injections, 3) post IVT injections complications like endophthalmitis (a sight-threatening infection), subconjunctival hemorrhage, raised intraocular pressure and rhegmatogenous retinal detachment *etc.* In addition, repeat IVT treatment posed a significant burden to patients and the healthcare system; for example overall annual cost of AMD care in the United States was estimated at $575 million in 2004 prior to the widespread use of anti-VEGF IVT injections and is projected to increase to $845 million over the next 15y[8-11].

Endeavors in ocular medicine has encouraged the ocular research community to not only focus on the development of novel anti-VEGF agents, but to also develop novel sustained drug delivery systems that increase the duration of action for currently available therapies. This is applicable to IVT administration as well as other routes like topical, trans-conjunctival, trans-scleral or suprachoroidal[12-14]. This approach helps to reduce the frequency, and consequently the risks related to multiple IVT injections and post injection complications like endophthalmitis. The bigger obstacle however to the discovery and development of improved therapies for retinal vascular diseases is the lack of animal models with larger eyes that can mimic the chronic phenotype of human ocular vascular diseases.

Currently available animal models that can be used to test the duration of action of newly developed therapies have some limitations such as a short efficacy window or retinal ocular vascular pathologies which heal rapidly over time[5-7,15]. It has been shown that post laser treatment in the laser induced choroidal neovascularization (CNV) model, that VEGF levels reach a peak on day 5 but decline quickly thereafter causing the CNV lesion to completely heal by day 14[5,15]. The VEGF induced retinopathy model has retinal vascular leakage which peaks on day 3 but returns to baseline levels on day 7 post VEGF IVT injection, resulting in the requirement for repeat IVT injections of active VEGF which in turn may cause post IVT injection related complications[7-9]. In the current study, we have developed and characterized a DL-2-aminoadipic acid (DL-AAA) (retinal glial cell toxin)[16-19] induced retinopathy model in pigmented Dutch Belted rabbits and evaluated the duration of action of currently available anti-VEGF and anti-inflammatory drugs over a period of 2mo. The DL-AAA rabbits were tested for sustained vascular leakage over 36mo with fluorescein angiography (FA). Additionally, a novel FA grading system was developed to enable accurate and consistent comparison between the drug's efficacy and it's duration of action. In summary, we demonstrated that the DL-AAA rabbit model represents the most ideal chronic model of retinal vascular leakage that can be utilized to test and develop novel therapies for ocular vascular diseases over an extended period of time.

MATERIALS AND METHODS

Ethical Approval All animal experiments adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The project was supervised by the Institutional Animal Care and Use Committee (IACUC) at Absorption Systems, a Pharmaron company, San Diego (CA, USA).

Animals Thirty-eight naïve (19 male and 19 female) Dutch Belted (*Oryctolagus cuniculus*) rabbits, approximately 1.5 to 2.5 kg, were purchased from Western Oregon Rabbit Company (OR, USA). Animals were acclimated for 10d before the enrollment on the study. All animals were maintained with a room temperature between 18℃ and 26℃, a relative humidity between 30% and 70%, and a 12-hour light-dark cycle in-house under pathogen-free conditions.

Animal Preparation and Anesthesia Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (20 mg/kg) and xylazine (5 mg/kg) prior to surgical procedures. Prior to test/control articles IVT administration, pupils were dilated with topical application of one drop each of 10% phenylephrine and 1% tropicamide on each cornea. A 5% Betadine solution was used to clean the eye and surrounding area. Betadine was applied for 5min, after which the eye was rinsed with balanced salt solution (BSS). After the area was surgically prepared, one to two drops of topical 0.5% proparacaine hydrochloride anesthetic were applied to the animal's eyes. After surgical procedure, atipamezole hydrochloride (1 mg/kg via intramuscular injection) was used as a reversal agent and after full anesthesia recovery animal received one injection of buprenorphine (0.02 mg/kg via subcutaneous injection). All *in vivo* imaging procedures were performed without anesthesia.

DL-AAA Preparation The 80 mmol/L DL-AAA solution (Sigma-Aldrich Corp.) was freshly prepared based on published procedures[16-19]. IVT injections were performed in 2 staggers (Stagger 1:10 males and 9 females; stagger 2:9 males and 10 females). In brief, 120 mg of DL-AAA was dissolved in 1 mL 1 mol/L hydrochloric acid (HCl) to create a stock solution. The components were gently swirled for 5min until a clear solution formed. For IVT administration, DL-AAA stock solution was diluted by adding 4 mL of 0.9% sterile normal saline solution, followed by sufficient 1 mol/L sodium hydroxide (NaOH) to adjust the pH of the solution to 7.4. Sufficient volume of saline was then added to bring the DL-AAA concentration to 80 mmol/L, the pH was re-tested, and a minimal volume of NaOH (4-5 μL) was added to bring the pH back to 7.4. The solution was then sterile filtered through a disposable 0.2 μm syringe filter to remove any potential particulates. DL-AAA solution was administrated within 15min of formulation preparation. Solutions were kept at room temperature until injection.

Pre-induction Clinical Ophthalmic Examinations and Randomization of Animals Prior to placement on study, each animal underwent clinical ophthalmic examinations (slit-lamp biomicroscopy and indirect ophthalmoscopy) and ocular findings were scored according to a modified McDonald-Shadduck Scoring System[20-22]. The acceptance criteria for placement on study was scores of "0" for all ocular variables. All animals were assigned to one experimental groups based on body weight for DL-AAA induction for retinal leakage. Post 12wk of DL-AAA induction, 20 animals were assigned a numeric rank from 1 to 20 according their FA scores in a decreasing order (animal with highest FA score was assigned rank =1) into four groups (Table 1).

Induction of Retinal Leakage *Via* Intravitreal Administration of DL-AAA A 31G beveled needle attached to 0.3 mL insulin syringe was inserted (right eye approximately 11 o'clock position and left eye approximately 1 o'clock) 3-4 mm away from the limbus into the vitreous body and 80 μL of the 80 mmol/L DL-AAA solution was administered into the mid vitreous. The needle was removed slowly to reduce risk of back flow from the injection track, and the eye was rinsed with BSS. Triple antibiotic ophthalmic ointment was administered to all eyes after dosing.

Anti-VEGF and Anti-Inflammatory Drugs Intravitreal Administration Once stable retinal leakage (12wk post DL-AAA induction) was established in rabbits, following the IVT injection procedure described in the previous section, the right eye was injected with either 40 μL of bevacizumab (solution 25 mg/mL, Genentech), 100 μL of ranibizumab (10 mg/mL, Genentech), 25 μL of aflibercept (solution 40 mg/mL, Regeneron) at a dose of 1 mg/eye or triamcinolone (TAA; 40 mg/mL, Bristol-Meyers Squibb) at a dose of 2 mg/eye (Table 1). The contralateral eye of each animal received equal volume of BSS. All drugs were delivered into the mid vitreous cavity.

Table 1 Concentrations and dosage of the drugs

Group	Treatment (OD)[a]	Concentration (mg/mL)	Dose (μL/eye)	Dose (μg/eye)
1	Aflibercept	40	25	1000
2	Bevacizumab	25	40	1000
3	Ranibizumab	10	100	1000
4	TAA	40	50	2000

[a]Contralateral eye (OS) received equal volume of balanced salt solution (BSS).

Fluorescein Angiography FA was performed using Heidelberg Retina Angiograph (HRA) device from Heidelberg Engineering (Heidelberg, Germany), the Spectralis ophthalmic imaging system on both eyes of all animals at baseline (prior to DL-AAA administration) and on weeks 2, 4, 6, 8, and 12 post DL-AAA administration. Additional FA was performed on days 7, 30, and 60 post IVT administration of bevacizumab, ranibizumab, aflibercept and TAA. In brief, sodium fluorescein was injected intravenously (IV) and FA time-course images were captured on both eyes between 30s to at least 10min post fluorescein injection. Laser intensity was kept at constant (55%) to avoid any overexposure of FA images. FA images were captured in three areas for retinal vascular leakage: optic nerve head, nasal optic streak, and temporal optic streak. In addition to leakage, any other associated pathology that was secondary to the retinal leakage (such as hemorrhage and retinal detachment) was also imaged. A novel grid based FA grading system was developed based on the published FA scoring system on monkey and rabbits and used for retinal vascular leakage grading[15,22-24].

Infrared, Color Fundus, and Optical Coherence Tomography Imaging Infrared (IR) and color fundus imaging were performed at baseline and 2wk post DL-AAA administration. Spectral-domain optical coherence tomography (SD-OCT) imaging was performed using HRA-OCT device from Heidelberg Engineering (Heidelberg, Germany), the Spectralis ophthalmic imaging system on both eyes of all animals at baseline (prior to DL-AAA administration) and on weeks 2, 4, 6, 8, and 12 post DL-AAA administration. In brief, OCT sessions were taken on superior/inferior/nasal/temporal and center retinal at 55° field of view using the high-resolution mode (signal quality ≥24 dB) with scan speed of 40 000 A-scans per second. The image scaling x and z were 1.10 μm per pixel and 3.87 μm per pixel, respectively. The optimal focus depth was approximately 3 diopters. Axial resolution was 7 μm optical and 3.5 μm digital. SD-OCT data were exported as 8-bit grayscale image.

Statistical Analysis Statistical analysis was performed by using the GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Percent of FA scores was compared and plotted among treatments groups. Data was presented as mean±standard error of the mean (SEM).

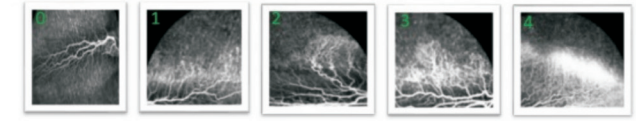
RESULTS

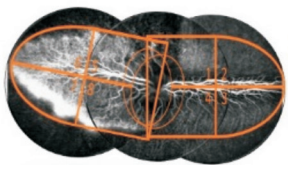
Clinical Ophthalmic Examinations Animals with no ocular anomalies, as examined and confirmed by slit-lamp biomicroscopy and indirect ophthalmoscopy, were enrolled onto the study. Ocular discharge and hyperemia was observed after IVT administration of DL-AAA, anti-VEGF and TAA on dosing day but resolved in most animals by the next day, confirming that they were related to IVT injection procedure. Occasional recurrences of ocular discharge were observed in a subset of animals, along with, more rarely, instances of ocular swelling. These symptoms of ocular irritation were likely due to underlying ocular inflammation. Post 12wk of DL-AAA administration, all the animals were presented with moderate to severe retinal/choroidal inflammation and were accompanied by faint to intense vitreous flare and sparse to numerous cells in the vitreous humor (VH). These findings likely reflect infiltration of inflammatory cells into the VH subsequent to persistent posterior inflammation and retinal hemorrhage. A subset of eyes also exhibited posterior lens opacities, likely due to cells from the vitreous space precipitating onto the posterior lens capsule. Sluggish pupillary response was noted in a subset of eyes, was likely also a result of persistent ocular inflammation. Eyes treated with 1 μg/eye of bevacizumab, ranibizumab, aflibercept and TAA had mild inflammation accompanied by very mild vitreous flare and low number of inflammatory cells into the VH, however, controls eyes treated with BSS had moderate to severe retinal/choroidal inflammation with intense vitreous flare and presence of significant number of cells in the VH, suggesting suppression of inflammation and vascular leakage post bevacizumab, ranibizumab, aflibercept and TAA administration. Ocular anomalies were significantly low by day 30 in the eyes treated with bevacizumab, ranibizumab, and aflibercept. However, TAA treated eyes had lowest level on day 7.

Novel Fluorescein Angiography Grading System for DL-AAA Rabbit Model A novel FA grading system was developed for grading of DL-AAA rabbit vascular leakage. FA images were exported as .jpeg files. FA images comprised of central, nasal and temporal retinal vasculature were aligned and 4 grids were placed on temporal (R1, R2, R3, and R4) and nasal (R5, R6, R7, and R8) retinal vasculature areas. At each time point vascular leakage was graded using following criteria, Grade 0: Major vessels straight some tortuosity of smaller vessels, no vessel dilation; Grade 1: Increased tortuosity of major vessels and/or some vessel dilation; Grade 2: Leakage between major vessels, significant vessel dilation; Grade 3: Leakage between major and minor vessels, minor vessels still visible; Grade 4: Leakage between major and minor vessels, minor vessels poorly/not visible (Figure 1). A total score consisting of the sum of all individual region scores was calculated for each eye at each time point.

FA Grading System for DL-AAA Animal Model

Score	Descriptor
0	Major vessels straight some tortuosity of smaller vessels, no vessel dilation
1	Increased tortuosity or neovessels with no or very slight leakage and clear background
2	Increased tortuosity or neovessels with slight leakage and clear background
3	Moderate leakage from all or some neo vascular vessels with visible background
4	Severe leakage from all neovessels with poorly/not visible background



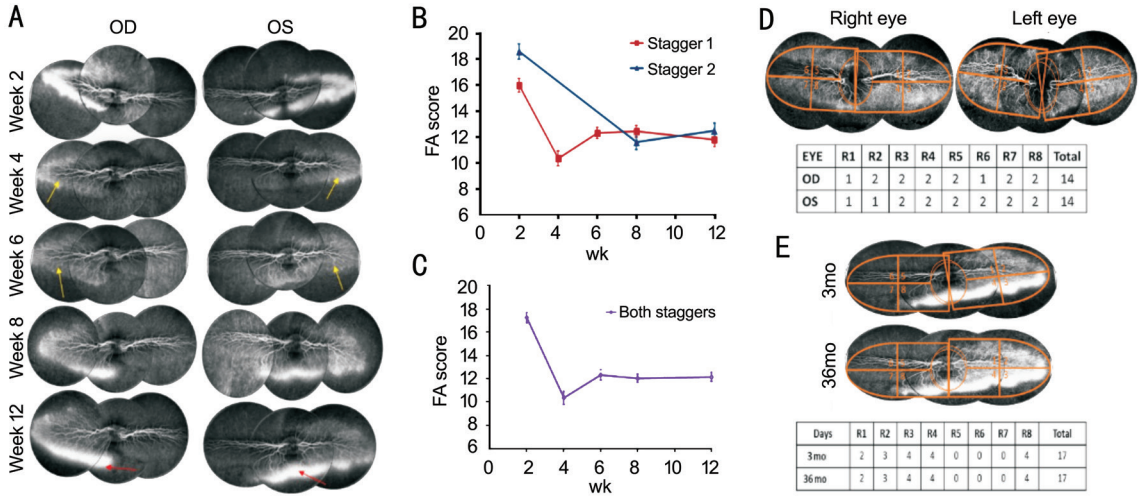


4101	R1	R2	R3	R4	R5	R6	R7	R8	TOTAL
OD	0	0	0	0	3	3	4	3	13
OS	0	0	2	4	0	0	0	2	8

Figure 1 Novel grid based FA grading system for DL-AAA rabbit model Top panel shows grading of retinal vascular leakage. Lower panel shows a typical example of FA grading and scores of DL-AAA rabbit eye.

Angiographic Features of DL-AAA Model FA scores reflected substantial vascular leakage in all the eyes of 38 animals after DL-AAA injection. The retinal vascular leakage observed in week 2 was accompanied by retinal hemorrhage and the growth of retinal neovessels (RNV). Neovessels continued to extend in weeks 4 and 6 in all eyes assessed at these time points. By week 8, retinal neovessels in all eyes remained tortuous and dilated and continued to extend. Vessels were still tortuous and dilated in week 12; the robust vascular leakage observed at this time point was now restricted to the tips of the telangiectatic neovessels (Figure 2A, red arrows). Temporal regions [regions 5-8 in right eyes (OD), regions 1-4 in left eyes (OS)] were more severely affected, while nasal regions (regions 1-4 OD, regions 5-8 OS) were affected less (Figure 2A). Notably, the injection site was located temporally (11 o'clock position OD, 1 o'clock position OS) in all eyes. Interestingly, in an additional cohort of rabbit (n=10), we demonstrated that homogeneous (both temporal and nasal retinal vessels) retinal leakage can be achieved by delivering DL-AAA *via* two 40 μL of 80 mmol/L DL-AAA injections per eye, 15min apart, one in each superior or inferior hemisphere (temporal at 11 or 8 o'clock position and nasal at 1 or 4 o'clock position; Figure 2D).

Maximum retinal vascular leakage was observed at 2wk after DL-AAA injection. In stagger 1 animals, a reduction in mean



EYE	R1	R2	R3	R4	R5	R6	R7	R8	Total
OD	1	2	2	2	2	1	2	2	14
OS	1	1	2	2	2	2	2	2	14

Days	R1	R2	R3	R4	R5	R6	R7	R8	Total
3mo	2	3	4	4	0	0	0	4	17
36mo	2	3	4	4	0	0	0	4	17

Figure 2 FA and quantification A: FA shows maximum retinal vascular leakage at 2wk post DL-AAA dose. Vascular leakage decreased to lower levels by 6wk (yellow arrows), and then increased again by 8wk to a level that was below the initial maximum, but still substantial. These levels of retinal vascular leakage then remained stable from 12wk post-dose and vascular leakage was restricted to the tips of the telangiectatic neovessels RNV (red arrows). Vascular leakage was most severe in the regions nearest the DL-AAA injection side. Vascular dilation and RNV and were more prominent in area centralis region (red arrows). B: Mean FA scores for stagger 1 and stagger 2. C: Combined mean FA scores of both eyes (stagger 1 and stagger 2). D: Homogeneous retinal leakage in an animal that received 2 IVT injections of DL-AAA. E: FA shows that retinal vascular leakage is consistently stable up to 36mo. Data as mean±SEM.

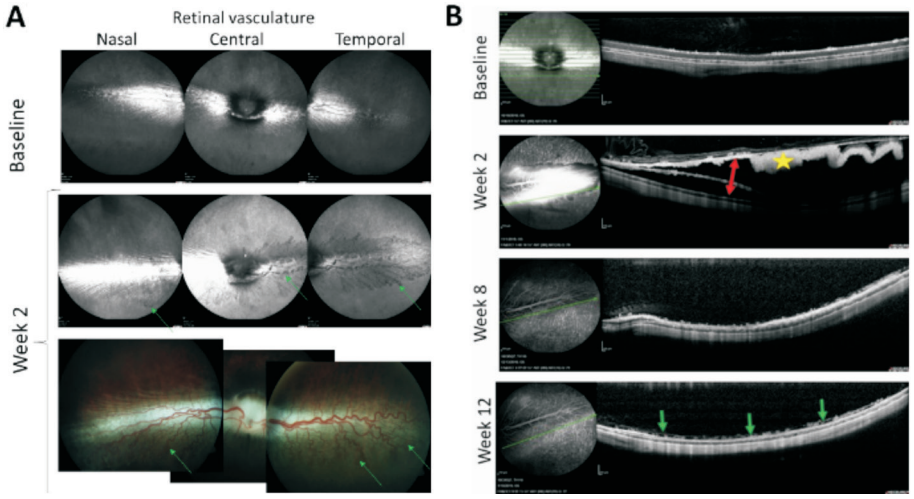


Figure 3 Funduscopic and tomographic features of DL-AAA model A: IR and color fundus imaging on week 2 after DL-AAA IVT injection, all the animals had tortuous vessels and growing of new epiretinal vessels towards retinal periphery and area centralis (green arrows); B: OCT imaging at baseline revealed normal retina. However, DL-AAA IVT injected animals showed severe intraretinal separation (red arrows) and retinal hemorrhage (yellow star) at week 2 post DL-AAA dose. Retinal degeneration was evidenced in the detached areas. Progressive degeneration of the retinal ganglion cell layer was observed from week 4 to 6. By week 8, retinal degeneration and thinning of retina in the detachment areas had progressed further, and by week 12, the retinal neuronal layer in these areas had completely disappeared (green arrows).

FA scores was observed in week 4; mean FA scores then increased again slightly, reaching a level that was also reduced from maximum values in week 2, but still showed robust leakage. This level of leakage then persisted as evidenced by stable mean FA scores in weeks 8 and 12. Stagger 2 animals were not assessed *via* FA at weeks 4 and 6, but showed comparable FA mean scores in weeks 8 and 12, *i.e.*, week 8 mean FA values that were reduced from the high values in week 2, but still reflective of robust leakage, and that remained stable at this level in week 12 (Figure 2B, 2C). A cohort of DL-AAA animals (n=10, received either 1 or 2 DL-AAA injections) was screened with FA at 36mo post DL-AAA induction. All the animals presented with same level of retinal vascular leakage suggesting that DL-AAA rabbit model consistently has stable vascular leakage up to 36mo (Figure 2E).

Funduscopic and Tomographic Features of DL-AAA Model IR and color fundus imaging showed that all animals had tortuous vessels, retinal hemorrhage (Figure 3B, yellow

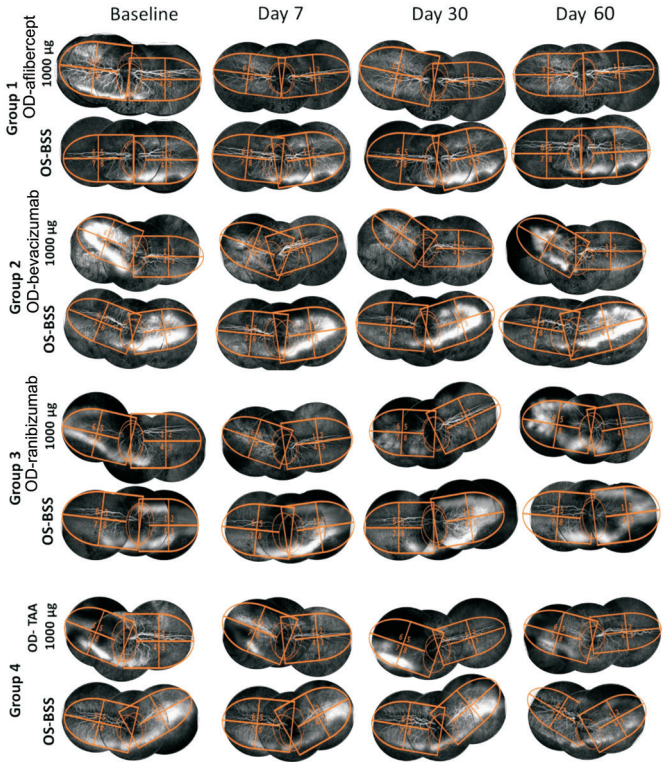


Figure 4 Efficacy of anti-VEGF and anti-inflammatory drugs in DL-AAA rabbit model FA shows complete suppression of retinal vascular leakage in the eyes (OD) treated with anti-VEGF drugs on day 7. TAA (Groups 4 OD) treatment also showed suppression of vascular leakage on day 7. Day 56 angiogram showed that vascular leakage levels returns to the baseline (Group 2 and 3 OD) for treated eyes following drug elimination, indicating the reversible nature of vascular leakage of the DL-AAA model. Aflibercept treated (Group 1 OD) eye angiogram showed substantial suppression of leakage for 60d. The control (OS) eyes treated with BSS in all groups did not show any change in vascular leakage though out the study.

star) and growth of new epiretinal vessels towards retinal periphery and area centralis within 2wk post DL-AAA IVT injection (Figure 3A, green arrows). Baseline before DL-AAA dose, OCT scans confirmed normal retina in all study animals. Week 2, OCT scans showed moderate to severe intraretinal separation with retinal degeneration (Figure 3B, red arrow) evidenced in the detached areas. Progressive degeneration of the retinal ganglion cell layer was observed in weeks 4 and 6 on OCT scans. By week 8, retinal degeneration and thinning in the detachment areas had progressed further, and by week 12, the retinal neuronal layer in these areas had completely disappeared (Figure 3B, green arrows).

Efficacy of Anti-Vascular Endothelial Growth Factor and Anti-inflammatory Drugs in DL-AAA Rabbit Model On day 7, mean FA score showed that 1 µg/eye of bevacizumab, ranibizumab, aflibercept suppress 65%, 90%, and 100% retinal vascular leakage, respectively, however, TAA 2 µg/eye showed only 50% suppression of vascular leakage.

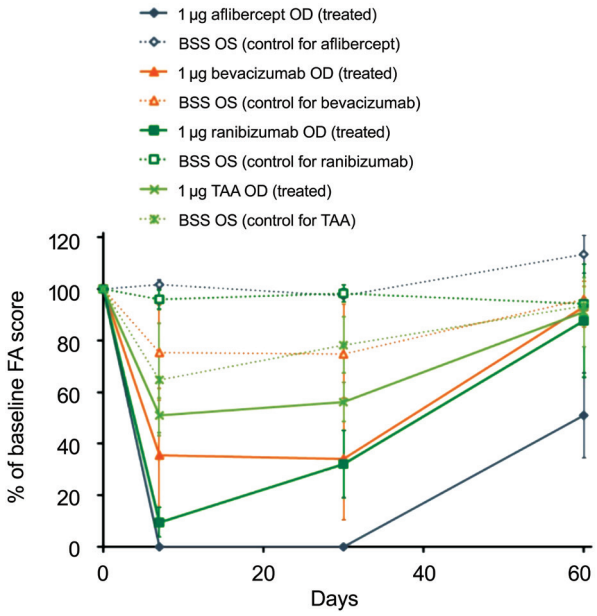


Figure 5 Quantification of FA scores The 1 µg of aflibercept, bevacizumab and ranibizumab suppressed retinal leakage. Aflibercept was most effective and completely suppressed leakage for 30d. At day 60, aflibercept was still effective and suppressed (50%) of leakage. Bevacizumab and ranibizumab suppressed leakage (70% for 30d) and returned to baseline leakage by day 60 post injection. TAA treatment showed maximum suppression on day 7 post IVT treatment. Data shown as mean±SEM. *n*=5 for each treatment and controls.

Day 30, bevacizumab and TAA continued to show 66% and 44% suppression of vascular leakage while ranibizumab effect was becoming less effective (68%) and reached a similar level as bevacizumab. In contrast, 1 µg/eye aflibercept was still able to fully (100%) suppress vascular leakage on Day 30. On day 60, bevacizumab, ranibizumab and TAA showed suppression of 7%, 12%, and 9% retinal vascular leakage, respectively, however, aflibercept continued to be more effective showing 50% suppression of vascular leakage on day 60. The day 60 angiogram showed that vascular leakage levels returned close to the baseline for bevacizumab, ranibizumab and TAA animals which is consistent with drug elimination, while also indicating the reversible nature of vascular leakage of the DL-AAA model. Control (OS) eyes treated with BSS in all groups didn't show any change in vascular leakage though out the study, suggesting that there was no crossover effect with either treatment (Figures 4 and 5).

DISCUSSION

We have established and validated a rabbit model with stable and chronic retinal vascular leakage, induced *via* IVT administration of DL-AAA, a glutamine synthetase inhibitor and retinal glial (Müller) cell toxin. Retinal Müller cells play an essential role in regulating neuronal activity and maintaining the integrity of the blood-retinal barrier[25-26]. The mechanism by which DL-AAA causes retinal disruption and

neovascularization development are not well understood. After IVT delivery, DL-AAA mainly target and damage the connecting bridges between retinal nerves cells and blood vessels (blood-retinal barrier), which causes metabolic dysfunction of retina, ischemia and retinal inflammation[27-29] and resulted an increase in VEGF levels in the retina. Higher levels of VEGF in the retina increase retinal vascular permeability and induced chronic condition of RNV[25-29]. IVT delivery of DL-AAA in primates, rodents and rabbits has been shown to induce RNV[16-19,29-30]. In a recent report, pigmented rabbit are shown to develop persistent and measurable RNV at a greater success rate, compared to non-pigmented rabbit[29]. In this study, IVT injection of 1.03 mg/eye DL-AAA in pigmented Dutch Belted rabbits was associated with substantial retinal vascular leakage, moderate to severe intraretinal separation with retinal degeneration in the areas of detachment, moderate to severe retinal/choroidal inflammation, retinal hemorrhage, tortuous and dilated retinal vessels, and RNV with epiretinal vessels growing towards the retinal periphery and area centralis. RNV was progressive, with neovessels continuing to extend for at least 8wk after DL-AAA injection. Retinal degeneration in the areas of detachment was likewise progressive, with continually more severe retinal thinning and degeneration of the retinal ganglion cell layer, culminating in degeneration of the optic streak and a complete disappearance of the retinal neuronal layer in the detachment areas by 12wk post-dose. However, these features do not exactly mimic all the pathological and genetic features of ocular vascular diseases, for example, DL-AAA rabbits are not presented with CNV like AMD or retinal edema like diabetic macular edema (DME). Nonetheless, the key pathognomonic feature of DL-AAA rabbit is RNV and retinal vessels leakage which is the most striking feature of most ocular vascular diseases[1-4]. DL-AAA model had vascular leakage at maximal 2wk after DL-AAA injection, decreased to lower but still robust levels by 4wk post-dose, and then increased again slightly to a level that was below the initial maximum, but still substantial by 6wk post-dose and finally becomes stable at 12wk post DL-AAA administration. Li *et al*[17] and Cao *et al*[18] observed vascular leakage up to 18 and 12mo, respectively, in a DL-AAA rabbit model. In this study, we observed levels of vascular leakage remained stable and restricted to the tips of the telangiectatic neovessels up to 36mo. Furthermore, consistency in developed retinal phenotypes and stable FA scores up to 36mo among all the animals, in two different staggers, showed that DL-AAA rabbit is an excellent preclinical model to test the long term drug efficacy for vascular inflammation and leakage.

Presence of vitreous flare, inflammatory cells in the VH, retinal inflammation including retinal hemorrhage and posterior lens capsule opacities did not interfere with *in vivo* imaging, and show that *in vivo* imaging in combination with clinical exams can provide long term efficacy evaluation of drugs that are designed to target vascular inflammation and leakage. No effect of the test article on body weights was observed during the study suggesting no systemic burden or toxicity in this experimental model.

Despite the fact that DL-AAA model does not mimic all the features of existing human retinal diseases, but, it still presents features like stable and persistent chronic retinal neovascular pathology and leakage similar to human vascular disease like nAMD, DME, and retinal vein occlusion[1-4]. Furthermore, higher vitreous VEGF levels in DL-AAA eyes compared to naïve animals[18] and longer duration of stable vascular leakage compared to other available animals models like laser induced CNV rodent model, are valuable features of DL-AAA rabbit model. The rabbit's large eye size with similar anatomical and physiological characteristics to human, is also well suited to test the efficacies of novel formulations and delivery of ocular devices. Therefore, collectively the described features of this experimental DL-AAA rabbit model, provide wide applicability making this model a valuable tool for the investigation of efficacy and duration of novel treatment strategies for retinal angiogenic diseases.

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Authors' contribution: Kumar S designed and performed experiments, analyzed data, supervised the study, and wrote the manuscript; Quach J, Cook N performed fluorescein angiography; Gum G helped with the clinical ophthalmic examinations; Naageshwaran V, Gum G supported the overall operations of the project, review and comments on the manuscript.

Conflicts of Interest: Kumar S, None; **Quach J,** None; **Cook N,** None; **Gum G,** None; **Naageshwaran V,** None.

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