Intravitreally Injected Fluid Dispersion: Importance of Injection Technique

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PURPOSE. The purpose of this study was to evaluate the dispersion of intravitreally injected solutions and investigate the influence of varying injection techniques.

METHODS. This was a prospective study using enucleated porcine eyes and ultra-high-resolution computed tomography (UHRCT) scanning to visualize iomeprol intravitreal dispersion. Sixty eyes were divided over 12 different groups according to the injection procedure: fast (2 seconds) or slow (10 seconds) injection speed and needle tip location (6- and 12-mm needle shaft insertion or premacular tip placement verified by indirect ophthalmoscopy). For each of these combinations, eyes were either injected with the combination of V20l (which is an analogue of ocriplasmin) and iomeprol or iomeprol alone. Distance to the macula and volume measurements were performed at 1, 2, 3, and 5 hours after injection.

RESULTS. The measured contrast bolus volume increases slowly over time to an average of 0.70 (P = 0.03), 1.04 (P = 0.006), and 0.79 (P = 0.0001) cm³ 5 hours after the injection for the 6-mm needle shaft insertion, 12-mm needle shaft insertion, and premacular needle tip placement, respectively. The distance to the macular marker was significantly lower for premacular needle tip placement injections compared with 6- and 12-mm needle shaft insertion depths.

CONCLUSIONS. Ultra-high-resolution computed tomography with three-dimensional reconstruction offers the possibility to study the dispersion of intravitreally injected solutions in a noninvasive manner. Intravitreal premacular solution delivery is possible with an indirect ophthalmoscope-guided injection technique and significantly reduces the time to reach the posterior pole in respect to 6- and 12-mm needle insertion depths. The speed of injection does not influence dispersion significantly.

Keywords: intravitreal injection, dispersion, vitreous, ocriplasmin

Intravitreal injections (IVIs) are currently one of the most commonly performed procedures in ophthalmology.1 Usually injections are made by inserting a thin needle (27 to 30 G) through the pars plana, at 3.5 to 4 mm from the limbus, inside the vitreous body.2 There is a wide variation in how far the needle shaft is inserted inside the eye, how fast the plunger is pressed, how the bevel is oriented, and what the angle is between the shaft and the pars plana.3 Potential concerns are the insertion depth of the needle and the time the injected drug needs to reach the posterior pole. In general, for most drugs, there is little clinical impact because the drug that is injected retains its activity for several weeks.4–8 In such a time frame, there is little clinical impact because the drug that is injected needs to reach the posterior pole. In general, for most drugs, there is little clinical impact because the drug that is injected retains its activity for several weeks.4–8 In such a time frame, there is little clinical impact because the drug that is injected needs to reach the posterior pole.

However, there are drugs that are short acting and would benefit from a targeted delivery such as ocriplasmin. Ocriplasmin (Jetrea; Alconn-Couvreur nv, Puurs, Belgium) is a recombinant protein representing a truncated form of the human serine protease plasmin which retained its full proteolytic activity.10,11 It cleaves various proteins inside the vitreous like laminin and fibronectin. Intravitreal administration of ocriplasmin is approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for the treatment of vitreomacular traction (VMT), including a macular hole of ≤400 µm or symptomatic vitreomacular adhesion (sVMA), respectively.12 However, after its commercial availability, clinical success rates of traction release varied extensively between different centers, which could indicate that the injection technique during administration of the drug is important.13 Ocriplasmin shows significant autolysis, retaining only 15% of its activity after 6 hours.14 Therefore, it may be important to deliver the molecule in close proximity to the desired location because waiting for drug diffusion throughout the vitreous will significantly decrease concentration of active drug at the targeted site.

Visualization of the dispersion pattern of intravitreally injected drugs is challenging. This has previously been studied either by using Indian ink or fluorescein.15–17 Both colorants are easily visualized in the vitreous cavity, but it is difficult to assess or measure the real size, shape, and dispersion rate of the contrast bubble by ophthalmoscopy. Furthermore, follow-up over a period of hours or days requires in vivo tests because the...
cornea and lens will hinder visualization in enucleated eyes. Others have tried to bypass this problem by carefully removing the vitreous as a whole and assessing the dispersion pattern. These manipulations might significantly influence the dispersion within the vitreous body.

This study aims at characterizing the intravitreally injected fluid dispersion pattern with the use of the contrast agent iomeprol combined with ultra-high resolution computed tomography scanning (UHRCCT, 0.3-mm collimation; Siemens Somatom Definition Flash, Erlangen, Germany) and subsequent three-dimensional (3D) image reconstruction in enucleated porcine eyes without removing the vitreous body. Additionally, different injection procedures will be compared to identify an alternative method for more targeted and efficient drug delivery.

METHODS

Injected Solutions

A radio-opaque water soluble contrast agent iomeprol (Iomeron 350; Bracco, Milan, Italy) was used to assess dispersion inside the vitreous body as seen on 3D-reconstructed UHRCCT images. The contrast agent was dissolved and diluted either in a buffer consisting of balanced salt solution (BSS; Alcon, Puurs, Belgium) or in the combination of the balanced salt solution and a vitreolytic agent (referred to as V20I). V20I is a recombinant ocirplasmin analogue in which valine (V) in position 20 is substituted by isoleucine (I). Production and purification of V20I, activity measurements against the chymotrypsinogen substrate S-2251, and the measure of the rate of autolysis in porcine vitreous were performed as previously described. Activity against fibronectin was determined using Oregon Green 488-labeled fibronectin. Human fibronectin (cat. F2006; Sigma-Aldrich Corp., St. Louis, MO, USA) was labeled using a commercial labeling kit (cat. O-2-241; Thermo Fisher Scientific, Villebon-sur-Yvette, France), and V20I activity on the labeled fibronectin was monitored by measuring the increase in fluorescence (excitation/emission at 476 nm/524 nm) that resulted from the de-quenching of the probe as a function of time. Supplementary Table S1 shows that the hydrolytic activity of V20I is indistinguishable from wild-type ocirplasmin, although its rate of autolytic inactivation is lower.14,18 Activity against fibronectin was determined using Oregon Green 488-labeled fibronectin. Human fibronectin (cat. F2006; Sigma-Aldrich Corp., St. Louis, MO, USA) was labeled using a commercial labeling kit (cat. O-2-241; Thermo Fisher Scientific, Villebon-sur-Yvette, France), and V20I activity on the labeled fibronectin was monitored by measuring the increase in fluorescence (excitation/emission at 476 nm/524 nm) that resulted from the de-quenching of the probe as a function of time. Supplementary Table S1 shows that the hydrolytic activity of V20I is indistinguishable from wild-type ocirplasmin, although its rate of autolytic inactivation is lower. Every IVI with V20I contained 96 μg of the active enzyme, which is the equivalent of the dose administered to humans (125 μg ocirplasmin) corrected for the smaller volume of the porcine vitreous. As the average axial length of porcine eyes is similar to that of humans, a flatter cornea and thicker lens imply a smaller average vitreous volume (3.5 vs. 4.5 mL) for porcine versus human eyes, respectively. Some eyes were pretreated with V20I 24 hours before injection with iomeprol to mimic age-related syneresis in the human vitreous body.21

Pilot Experiments

First, a pilot experiment was performed to determine the optimal dilution of iomeprol to reduce scattering (assessed by averaging signal intensity around the contrast bolus with respect to noninjected vitreous body signal strength). In addition, optimum conditions for maintaining signal strength were studied to reduce the impact of iomeprol diffusion during monitoring of intravitreal dispersion. Enucleated porcine eyes were acquired from a nearby slaughterhouse (Eggen slaughthuis Mechelen NV, Mechelen, Belgium) as a byproduct of meat production for human consumption. Not a single animal was killed for this particular research, as all eyes used in this study were slaughterhouse waste. Five fresh enucleated porcine eyes were injected with 0.1-mL solutions containing 100%, 50%, 25%, 20%, and 10% iomeprol diluted with BSS.

The injection was made with a 30-G ½-inch needle (Terumo Europe NV, Leuven, Belgium), with the needle shaft completely inserted in to the eye aiming for the midvitreous. Consequently, the eyes were scanned by UHRCCT, and images were inspected for clear delineation of the contrast bubble, scattering, and intensity. A 10% (v/v) diluted mixture was selected because of optimal balance between persistent visibility and scatter.

Thereafter, a total of eight eyes were injected with iomeprol (four eyes) or the combination of iomeprol and V20I (four eyes) to serve as a baseline for sample size calculations. These eyes were scanned at 1, 2, 3, and 5 hours after injection. Three-dimensional-reconstructed images were made, and signal intensity assessed between the contrast bolus and the surrounding vitreous. A cutoff intensity of 180 Hounsfield Units (HU) proved to be most reliable in assessing volume changes of progressively diffused contrast, neither under- nor overestimating the extent of dispersion. This cutoff value was confirmed by assessing the maximal vitreous density (in HU) in nontreated eyes. Image analysis was performed using the built-in analysis software of the Siemens UHRCCT device. Volume computations were done manually by delineating the contrast bolus and measuring pixels between 180 and 3071 HU.

Contrast stability with and without the presence of V20I was tested by scanning small plastic containers (Eppendorf tubes; Eppendorf, Retselaar, Belgium) containing the same concentration and volume of the intravitreal injected solutions (Supplementary Fig. S1) at different time points. Furthermore, neither the enzymatic activity of V20I nor the contrast delivering capacity of iomeprol was altered when the two were combined and injected, as was confirmed by in vitro enzymatic activity tests.

To determine the region of interest (the macula), a marker (Gutta Percha points number BS080; Maxima by Henry Schein, Melville, NY, USA) was placed at 4.5 mm from the center of the optic nerve and fixed externally to bare sclera with medical glue (Histoacryl Tissue Adhesive; B. Braun, Rubi, Spain). To assess the optical pathway, a second marker was placed at the center of the cornea, as depicted in Figure 1: IVIs aiming for the midvitreous. Consequent-
study IVI to mimic the more liquefied vitreous of humans in respect to young porcine eyes.

Injection Technique

All injections, except the premacular IVIs, were done with a flow-controlled pump (Fig. 2). The volume for each IVI was set to 0.1 mL, and the time of plunger movement was either 2 or 10 seconds. A 30-G ½-inch needle was used for the centrally aimed injections, both with 6-mm shaft insertion and 12-mm shaft insertion. The needle shaft was always placed perpendicular to the globe following a 28° angle between the limbal plane and the needle axis according the setup displayed in Figure 2.

For the macular-oriented injections, a 30-G 1-inch needle (BD PrecisionGlide needles; PrecisionGlide, Franklin Lakes, NJ, USA) was used, and the injections were done manually by visualizing the needle tip’s position using indirect ophthalmoscopy holding a 20-diopter lens (Volk Optical, Mentor, OH, USA) in one hand and the syringe in the other hand. The needle was inserted through the pars plana at 3.5 mm from the limbus on the temporal side of the eye, which is the longest distance from the optic nerve. Given that the macular marker was placed externally 4.5 mm temporal from the center of the optic nerve and that porcine eyes lack a recognizable macula, the needle tip was advanced in the vitreous body in a straight line to about 3 disc diameters temporally from the center of the optic nerve. The shadow of the needle shaft was used to accurately determine preretinal needle tip position before the solution was injected (Supplementary Video S1). The injection speed for these manual injections was approximately 0.05 mL/s.

Statistical Analysis

A sample size based on a power of 80% and a type I error occurrence of 5% for two independent samples was calculated using the pilot experiment volumetric measurements 1 hour after injection comparing diluted iomeprol with the combination of iomeprol and V20I (mean contrast bolus volume of 0.48 ± 0.1 and 0.68 ± 0.07 mL, respectively). At least four eyes per group were needed to detect, with a power of 0.8 and a significance level of 0.05, a statistical significant difference in contrast volume of 0.2 mL 1 hour after the injection.

Nonparametric Mann-Whitney U tests for independent variables were used to compare between groups, and nonparametric Friedman tests were used to compare follow-up measurements within groups. Dunnett’s correction for multiple testing was applied.

RESULTS

In total, 60 fresh enucleated porcine eyes were injected with diluted iomeprol or a combination of iomeprol and V20I, according to the experimental design (Table 1). Table 2 depicts the volumetric measurements (average and SD) for each subgroup on the different imaging moments. The volume for the fast iomeprol injections (Groups 1, 6, and 11) increases slowly from 1 hour after the IVI to significantly larger average values of 0.70, 1.04, and 0.79 cm³ 4 hours later (P = 0.03, P = 0.006, and P < 0.0001, respectively). The 5-hour dispersed volume of iomeprol injected at 12 mm was significantly larger compared with the 6-mm injected contrast agent (comparing groups 6 and 1; P = 0.03). Adding V20I to iomeprol significantly increased iomeprol diffusion after injection at 6 mm, as the difference between the volumetric data of groups 1 and 2 show (P = 0.03). In contrast, there was no significant difference between the dispersion rates of diluted iomeprol (groups 6 and 11) versus the combination of iomeprol and V20I (groups 7 and 12, respectively) for the 12-mm and premacular tip locations (Fig. 3; Table 3). Pretreating the enucleated eyes with V20I to liquefy the central vitreous body did not influence dispersion rates significantly (comparing between groups 2 and 3 and between groups 7 and 8). Varying the injection speed between 2 and 10 seconds did not significantly influence volume measurements except for 6-mm injections, where slow (10 seconds) injections without
V20I (groups 1 vs. 4) showed a significant larger contrast bolus volume in contrast with the comparison between groups 2 and 5, where the fast (2 seconds) injection showed a trend toward a significant larger volume 1 hour after the injection ($P = 0.05$ and 0.06, respectively; Table 2).

Table 4 shows the distance to the macular marker in all groups with a fast (2 seconds) IVI. The distance to the macula measured 1 hour after the injection was significantly lower for premacular injected eyes (combining data from groups 11 and 12) compared with the 6-mm needle shaft insertion depth (data from groups 1 to 3) and the 12-mm (data from groups 6 to 8) needle shaft insertion depth ($P = 0.011$ and $P = 0.028$, respectively; Table 5). One hour after injection, 7 of 10 premacular injected eyes showed a direct overlying contrast bolus in respect to the macular marker (Fig. 4). Remarkably, premacular injected eyes showed a direct overlying contrast bolus in respect to the macular marker (Fig. 4). Remarkably, there were no significant difference in the distance measurement between the 6- and 12-mm injection depth methods (comparing groups 1 to 3 with groups 6 to 8), and the rate of reducing the distance from the edge of the contrast bolus to the macular marker was comparable. The 3D-reconstructed images showed that the shape of the contrast bolus is more elongated either toward the macular region or following the posterior lens curvature for 6-mm needle insertion depths compared with 12-mm insertion depth injections where a more round-shaped contrast bolus was observed (Fig. 5). This difference in shape explains the larger variation in measured distances in the 6-mm needle insertion depth groups versus the 12-mm needle insertion depth injections (SD of 4.50 vs. 2.72 mm at 1 hour, respectively; $F = 2.74$, $P = 0.55$).

**DISCUSSION**

Until now, different dyes (Indian ink and fluorescein) were used to investigate dispersion inside the vitreous body.\(^{15-17}\) Assessment of dispersion was done by ophthalmoscopy or by dissecting the vitreous carefully out of the eye. In this study, a new method was established using an UHRCT scanner and a radio-opaque contrast agent to visualize the dispersion in a 3D-reconstructed manner without any vitreous manipulation. This method of imaging decreases the possible bias induced by different manipulations eviscerating the vitreous body and renders 3D-reconstructed images with the possibility for volume and distance measurements in respect to in vivo tests with any colorant. The resolution of the UHRCT is high enough to clearly delineate the contrast bolus and assess its dispersion in the hours following an IVI.

Although the contrast agent iomeprom is water soluble and a small molecule (molecular weight, 777 Da), the dispersion rate in vitreous is relatively slow. This is illustrated by the recognizable shape of the injected bolus that lasts for several hours. On average, it takes more than 5 hours for the contrast bolus to enlarge to about 35% of the vitreous cavity volume. Unlike the young porcine vitreous, the middle-aged human vitreous is an inhomogeneous gel-like mass with several fluid-filled cavities as shown by Worst and Los.\(^{22}\) Therefore, the speed of diffusion of any molecule inside the vitreous cavity will vary according to the viscosity of the immediate surrounding tissue and could be influenced by convection.\(^{9}\) This is illustrated by the significant decreased half-life of intravitreally administered drugs after vitrectomy.\(^{23}\) To mimic the human middle-aged vitreous, a subgroup of the young enucleated porcine eyes was injected with the vitreolytic agent V20I 24 hours before injection with iomeprom. Interestingly, our results did not show a significant difference in dispersion rate between native and pretreated eyes.

Figure 4 shows the concave anterior shape of the iomeprom bolus according to the posterior lens curvature when injected at 6-mm needle insertion depth, 1 hour after injection, in comparison to another eye of the same group where the contrast was visualized in close contact with the peripheral retina. This could be due to the laminar arrangement of densely packed collagen fibers at the vitreous base.\(^{24}\) This fan-like composition of anterior fibers making contact with the posterior lens capsule and more posterior vitreous base-located fibers running toward the posterior pole might direct any injected solution either toward the lens or toward the posterior pole. The difference in dispersion pattern explains the large variation observed in the distance of the edge of the contrast bolus to the macular marker for 6-mm needle insertion depth IVIs. Together with the relatively slow observed dispersion rate, this implies that any medication that is injected at the vitreous base needs to travel for a significant time and distance before it reaches its point of action, for example, the macular region. It will make contact in a more concentrated form with the surrounding tissues first: namely the lens and zonule, the pars plana, and the peripheral retina.

Depending on the varying viscosity and convection currents inside the vitreous body and its lacunae, the surface area and dilution of the bolus of medication will increase asymmetrically in different directions. This could result in a large interpatient difference of exposure of any drug to a specific target inside the eye. Ocriplasmin not only degrades extracellular matrix protein such as laminin and fibronectin, but it also inactivates itself by autolysis. Therefore, the half-life in the vitreous is relatively short.\(^{14}\) As a last obstacle, the laminar posterior vitreous cortex has a high density and viscosity and is even strong enough to

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**Table 2.** Volumetric Measurements (Average ± SD in Milliliters, n = 5) of Iomeprin in Porcine Eyes Visualized by UHRCT at Different Time Points After Intravitreal Injection

<table>
<thead>
<tr>
<th>Group</th>
<th>1 Hour</th>
<th>2 Hours</th>
<th>3 Hours</th>
<th>5 Hours</th>
<th>$P$ Value, Friedman Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.53 ± 0.07</td>
<td>0.57 ± 0.11</td>
<td>0.60 ± 0.11</td>
<td>0.70 ± 0.14</td>
<td>0.0515</td>
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<tr>
<td>2</td>
<td>0.82 ± 0.15</td>
<td>0.92 ± 0.28</td>
<td>1.10 ± 0.22</td>
<td>1.20 ± 0.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>0.67 ± 0.09</td>
<td>0.66 ± 0.15</td>
<td>0.83 ± 0.17</td>
<td>0.75 ± 0.23</td>
<td>0.2096</td>
</tr>
<tr>
<td>4</td>
<td>0.74 ± 0.30</td>
<td>0.83 ± 0.40</td>
<td>0.88 ± 0.51</td>
<td>1.31 ± 0.27</td>
<td>0.0009</td>
</tr>
<tr>
<td>5</td>
<td>0.72 ± 0.11</td>
<td>0.70 ± 0.12</td>
<td>0.71 ± 0.13</td>
<td>0.90 ± 0.20</td>
<td>0.0196</td>
</tr>
<tr>
<td>6</td>
<td>0.76 ± 0.17</td>
<td>0.87 ± 0.24</td>
<td>0.92 ± 0.32</td>
<td>1.04 ± 0.32</td>
<td>0.0055</td>
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<tr>
<td>7</td>
<td>0.81 ± 0.14</td>
<td>0.83 ± 0.13</td>
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<td>1.00 ± 0.12</td>
<td>0.1616</td>
</tr>
<tr>
<td>8</td>
<td>0.90 ± 0.34</td>
<td>1.11 ± 0.45</td>
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<td>1.28 ± 0.62</td>
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<tr>
<td>9</td>
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<td>1.02 ± 0.19</td>
<td>1.10 ± 0.20</td>
<td>1.13 ± 0.16</td>
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</tr>
<tr>
<td>10</td>
<td>0.76 ± 0.12</td>
<td>0.78 ± 0.16</td>
<td>0.91 ± 0.19</td>
<td>1.01 ± 0.27</td>
<td>0.2096</td>
</tr>
<tr>
<td>11</td>
<td>0.58 ± 0.11</td>
<td>0.64 ± 0.12</td>
<td>0.76 ± 0.16</td>
<td>0.79 ± 0.12</td>
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</tr>
<tr>
<td>12</td>
<td>0.55 ± 0.21</td>
<td>0.66 ± 0.25</td>
<td>0.75 ± 0.29</td>
<td>0.97 ± 0.30</td>
<td>&lt;0.0001</td>
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</table>
cause vitreomacular traction. The premacular bursa is a fluid filled cavity inside the vitreous body that could be communicating via the space of Martegiani and Cloquets canal to the retrolenticular space.\textsuperscript{25} As diffusion and convection within these fluid-filled cavities will be much faster than in a gel-like vitreous, active molecules will be diluted and the concentration gradient to diffuse through the posterior vitreous cortex to reach the vitreoretinal interface will decrease. Taking the case of a product with a short half-life, this could explain the difference between a successful treatment or otherwise. Thus, with a conventional IVI, only a portion of the injected enzymatically active molecules will reach the macular region. Next to short-acting (enzymatic) drugs, retinal gene therapy with viral vectors could possibly also benefit from targeted intravitreal solution delivery. It seems important to expose the targeted retinal cells to abundant viral particles for successful DNA integration and transcription. As gene therapy is usually indicated in fairly young patients, the vitreous body and the injection technique could possibly play an important role in the dispersion of the viral vector.\textsuperscript{26,27} Targeted intravitreal solution delivery is to be regarded as significantly less invasive than

### TABLE 3. Statistical Analysis of the Influence of V20I and Injection Speed on Volume Changes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group Comparison</th>
<th>P Value, Mann-Whitney U Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iomeprol ( \leq ) V20I</td>
<td>1 vs. 2</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>4 vs. 5</td>
<td>0.2000</td>
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<tr>
<td></td>
<td>6 vs. 7</td>
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<td></td>
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<td></td>
<td>11 vs. 12</td>
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<tr>
<td>V20I pretreatment</td>
<td>2 vs. 3</td>
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<tr>
<td></td>
<td>7 vs. 8</td>
<td>0.0571</td>
</tr>
<tr>
<td>Injection speed</td>
<td>1 vs. 4</td>
<td>0.0286</td>
</tr>
<tr>
<td></td>
<td>2 vs. 5</td>
<td>0.0571</td>
</tr>
<tr>
<td></td>
<td>6 vs. 9</td>
<td>0.3429</td>
</tr>
<tr>
<td></td>
<td>7 vs. 10</td>
<td>0.8857</td>
</tr>
</tbody>
</table>

**Figure 3.** Graph on volume increment of iomeprol injection at 6 and 12 mm and premacular depth with or without V20I.

**Figure 4.** Ultra-high-resolution computed tomography scans 1 hour after injection of the eyes in group 11 and group 12 (both groups with premacular injections). The 3D-reconstructed images were rotated viewing from posterior (looking at the macular marker) to allow assessment of the location of the iomeprol bolus with respect to the marker, as shown in the schematic drawing. Note the overlying iomeprol in contact with the retinal surface (following the convex shape of the globe) directly located over the extraocular macular marker (bright white shape designated with M, measuring approximately 1 to 1.5 mm in length) in 7 of 10 eyes.
Group Comparison the Distance of Iomeprol to the Macula 1 Hour After Injection

<table>
<thead>
<tr>
<th>Group</th>
<th>1 Hour</th>
<th>2 Hours</th>
<th>3 Hours</th>
<th>5 Hours</th>
<th>P Value, Friedman Test</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>6.08 ± 4.50</td>
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<td>1.40 ± 1.67</td>
<td>0.80 ± 1.50</td>
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<tr>
<td>2</td>
<td>3.54 ± 5.00</td>
<td>0.90 ± 1.34</td>
<td>0.70 ± 1.10</td>
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<td>3</td>
<td>6.16 ± 0.99</td>
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<td>1.20 ± 1.10</td>
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<td>6</td>
<td>5.21 ± 2.72</td>
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<tr>
<td>7</td>
<td>5.56 ± 2.35</td>
<td>1.40 ± 1.14</td>
<td>0.20 ± 0.45</td>
<td>0.20 ± 0.45</td>
<td>&lt;0.0001</td>
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<td>8</td>
<td>4.33 ± 5.04</td>
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<td>11</td>
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<tr>
<td>12</td>
<td>0.86 ± 1.92</td>
<td>0.80 ± 1.79</td>
<td>0.20 ± 0.45</td>
<td>0.00</td>
<td>0.4446</td>
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</table>

TABLE 4. Measurements of Minimal Distance Between Iomeprol Bolus and Macular Marker (Average ± SD in Millimeters, n = 5) at Different Time Points After Intravitreal Injection Into Pig Eyes

TABLE 5. Statistical Analysis of the Influence of Injection Location on the Distance of Iomeprol to the Macula 1 Hour After Injection

<table>
<thead>
<tr>
<th>Group Comparison</th>
<th>P Value, Mann-Whitney U Test</th>
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<tr>
<td>1 vs. 6</td>
<td>0.8413</td>
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<tr>
<td>2 vs. 7</td>
<td>0.3095</td>
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<tr>
<td>3 vs. 8</td>
<td>1.0000</td>
</tr>
<tr>
<td>1-3 vs. 11 and 12</td>
<td>0.0112</td>
</tr>
<tr>
<td>6-8 vs. 11 and 12</td>
<td>0.0281</td>
</tr>
</tbody>
</table>

subretinal injections as, for instance, performed for the treatment of choroideremia.28

Premacular injections were carried out with a 24 mm 30-G needle using indirect ophthalmoscope guidance. The UHRCT images showed a direct placement over the predetermined macular region of the injected contrast in 7 of 10 eyes 1 hour after injection. Placing the marker on the outside of the globe and lacking a recognizable retinal macular area in porcine eyes, there was a researcher induced mismatch between the extracellular location of the marker and the intraocular preretinal needle tip for the three remaining eyes. This would not be an issue in the human eye as the macula is a clearly recognizable during ophthalmoscopy.

Asami et al.17 described the use of a quadraport needle with four openings on the side and an occluded tip to disperse the injected fluid faster inside the vitreous. Their tests with Indian ink showed a significantly larger dispersion when using this new needle in comparison to standard needles. The question, however, remains if, for instance for the treatment of VMT, it would be preferred to have the medication diluted throughout the vitreous body or a topical premacular administration rendering the highest concentration and activity at the vitreomacular adhesion site. This approach has been described and advocated by Sebag.24

In an effort to decrease dispersion time to reach the macular region while keeping the safety of a standard ½-inch 30-G needle, the study design comprised several groups with insertion of the full 12-mm needle shaft length into the eye. This hypothesizes that the time to reach the macular region would be much smaller compared with the conventional 6-mm shaft insertion depths. Our results indicated that there was no significant difference between the 6- and 12-mm needle insertion depth subgroups for the time to reach the macular region. The number of hours needed to diffuse throughout the vitreous cavity are clinically significant, taking into account the exponential decay of enzymatic activity of ocriplasmin.14 Performing an IVI under indirect ophthalmoscopy assures the clinician about needle tip position and the effect of the stream of fluid when the plunger is advanced. Interestingly, volumetric measurements showed significantly smaller initial volumetric values increasing at the same dispersion rate as the other intraocular needle tip positions. This implies optimal placement of the drug in its most concentrated form at the desired location of action.

Apparently, the speed of injection does not influence dispersion significantly as slow (10 seconds) and fast (2 seconds) IVIs resulted in the same diffusion rates. Manual injections can be done even faster generating more turbulence. However, it is important not to damage the retina by generating a high fluid flux. As a standard IVI is done in a blind manner, the clinician does not see the needle tip nor the jet of injected fluid. Premacular injections are better controlled because they are performed under indirect ophthalmoscope guidance. Actually visualizing the needle tip reassures the surgeon about position and intraocular effect of the fluid flux. Furthermore, pulsations at the optic disc can be monitored as injecting the product could increase the intraocular pressure to significant and harmful values.29

The results of this study should be interpreted with caution because changing the threshold (180 HU) for measuring the size of the contrast bolus would have profound effects on the volumetric measurements. As explained in the methods section, this arbitrary threshold was chosen as a cutoff value higher than the highest measured values in native vitreous. The results presented might thus be an underestimation of contrast dispersion. Therefore, the relative differences of volume increment or distance to the macula are more important and do reflect a difference in dispersion rate rather than the absolute values.

Additional limitations of this study consist of the use of young fresh enucleated porcine eyes and the imaging of a contrast agent instead of the drug itself. Although the contrast...
agent is a small water-soluble molecule, extrapolation and interpretation of these results regarding the intravitreal pharmacokinetics of enzymatic drugs should be made with caution. Monitoring could not be extended more than 5 to 6 hours because dilution of the contrast agent made a reliable analysis impossible. By then, the density of the edges of the bolus decreased to the same level as the surrounding vitreous.

The difference between young porcine vitreous and middle-aged partially liquefied human vitreous could have a profound effect on the results presented in this study. Although porcine vitreous does have the same molecular constitution as human vitreous, the lack of fluid-filled cavities in these young animals' eyes limits the process of dispersion largely to passive diffusion. In a nonhomogenous environment of gel-like vitreous and low-viscosity watery cavities, convection currents might significantly influence the process of dispersion (and dilution). To mimic partially liquefied (middle-aged) vitreous, a subset of eyes (groups 5 and 8) were injected with V20I 24 hours before the injection with iomeprol. This model of partially liquefied porcine vitreous probably does not represent the complex anatomy of the middle-aged human vitreous body. For instance, 20% of the vitreous body is already liquefied at the age of 4 years old, and this process will continue throughout life until less than half of the vitreous body remains gel-like at the age of 90.21 Furthermore, the vitreoretinal interface will undergo changes and the adhesion will weaken, ultimately leading to a posterior vitreous detachment.24 This process could be accelerated by intraocular surgery (for instance cataract surgery or [partial] vitrectomy), resulting in a different molecular environment with completely different pharmacokinetic properties.30 Therefore, future research investigating intravitreal pharmacokinetics should use human cadaver eyes to clarify the effect of natural age-related liquefied vitreous together with pseudophakic or vitrectomized eyes.

A second major limitation is that the effect of saccadic eye movements was not evaluated in this study nor were the eyes stored at body temperature. Pilot experiments, however, showed no difference in iomeprol dispersion when the eyes were placed on a shaking plate in between the measurements, and V20I-pretreated eyes stored at 37°C did not show a difference in iomeprol dispersion with respect to room temperature-stored eyes. Furthermore, all eyes were fixed within plastic containers with the optical axis oriented horizontally and parallel to each other to standardize injection and scanning angle. Mimicking the primary gaze position of the eye, the impact of gravity and geometry of the vitreous body would be similar to the upright position of the patients' head during the hours after injection. Because convection currents in partially liquefied vitreous could be tremendously influenced by rapid eye movements, future research should investigate the effect of the ocular orientation and rotating movements on the dispersion rate of intravitreally injected solutions.

Additionally, V20I has a similar pharmacodynamic profile as ocirplasmin (Supplementary Table S1), but additional data of mechanical tests are required to confirm effective V20I-induced vitreous body liquefaction effective mimicking human middle-aged vitreous.51 Furthermore, labeling the enzymatic molecules would render more direct and specific information about intravitreal pharmacokinetics.

In case of targeted vitreolysis to treat focal VMT, the local administration of highly concentrated and maximal effective molecules would be preferred. Our results do show that an indirect ophthalmoscope-guided premacular injection is possible and that the injected solution, in its highest concentration, can be delivered to the targeted site of action. A preliminary study on a surgical microscope-guided intravitreal injection of ocirplasmin in seven patients showed a significantly higher VMT resolution rate compared with nonguided conventional intravitreal injections, illustrating the importance of targeted drug delivery.32

**CONCLUSIONS**

Ultra-high-resolution computed tomography with 3D reconstruction offers the possibility to study the dispersion of intravitreally injected solutions in a noninvasive manner. Dispersion of small molecules inside the vitreous cavity is a relatively slow process. Intravitreal premacular solution delivery is possible with an indirect ophthalmoscope-guided injection technique using a 1-inch 30-G needle. Targeted intravitreal therapy could be of importance not only for short-acting drugs but possibly also for intraocular gene therapy.

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