Neovascularization Grading Methods in a Rat Model of Retinopathy of Prematurity

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PURPOSE. The method of counting cell nuclei above the internal limiting membrane in histologic sections is considered the standard when quantifying neovascularization (NV) in rodent oxygen-induced retinopathy (OIR). An alternative, more rapid method of counting clock hours in flat-mounted adenosine diphosphatase (ADPase)-stained rat retinas is analogous to clinically scoring retinopathy of prematurity (ROP). In the present study, the validity of counting clock hours was evaluated by a direct comparison of these techniques. The intereye correlation of NV score and retinal vascular area were also studied.

METHODS. Newborn Sprague-Dawley rats were exposed to cycles of $O_2$ (80–10%) for 7 days, followed by 5 days of room air recovery. Preretinal NV was quantified by three masked observers counting clock hours in flatmounted ADPase-stained retinas of both eyes. Retinal vascular and total retinal areas were calculated using computer-assisted analysis. Representative retinas that had been scored positive ($n = 10$) and negative ($n = 3$) for NV and room air control retinas ($n = 3$) were embedded in parafin. Each entire peripheral retinal quadrant was serially sectioned at 6 μm and stained with hematoxylin and eosin. Nuclei above the internal limiting membrane were then counted in a masked manner. The total number of nuclei counted per retina was defined as the nucleus count (704–938 sections per retina; 12,900 sections). Correlations were evaluated using Spearman rank coefficients.

RESULTS. The nucleus count was 0 to 44 in room air control retinas, 0 to 40 in negative OIR retinas, and 250 to 5634 in positive OIR retinas. The nucleus count was highly correlated with the clock hour score ($r_s = 0.95$, $P = 0.0001$). For the paired retinas, there was a significant correlation between right and left eyes in the severity of NV (clock hours; $r_s = 0.76$, $P = 0.0001$) and the ratio of retinal vascular area to total retinal area ($r_s = 0.81$, $P = 0.0001$).

CONCLUSIONS. The more rapid method of counting clock hours in flatmounted ADPase-stained retinas is valid for quantifying NV in rat models of ROP. Incidence and severity of NV and vascularized areas were similar between left and right eyes, which permits the use of paired retinas for complementary research techniques. (Invest Ophthalmol Vis Sci. 2000;41:887–891)

Retinopathy of prematurity (ROP) is a blinding disease of premature infants characterized by preretinal neovascularization. Oxygen-induced retinopathy (OIR) in the neonatal rat has been widely used as an animal model for ROP. In addition to OIR, exposure of neonatal rats to carbon dioxide or metabolic acidosis also results in preretinal neovascularization analogous to ROP. These models have been termed carbon dioxide–induced retinopathy (CDIR) and metabolic acidosis–induced retinopathy (MAIR), respectively. In all these models, a standard system of quantification of neovascularization is desirable. Previously described methods of quantifying neovascularization in animal models of ROP include counting or examining cell nuclei above the internal limiting membrane, counting mitotic cells within the nerve fiber layer, scoring vaso-obliteration, capillary tufts, and ridges, and counting clock hours of neovascularization scoring analogous to clinical assessment of ROP. Counting cell nuclei may be considered the most rigorous of these techniques, but it is the most time consuming. Counting clock hours in adenosine diphosphatase (ADPase)-stained retinas allows scoring of many more retinas in less time and is directly analogous to the commonly used clinical ROP grading method.

Models of ROP exhibit inherent variability analogous to clinical ROP; some retinas demonstrate severe preretinal neovascularization, whereas others have no or mild neovascularization. To study causative risk factors and potential interventions, the detection of small changes in severity is desirable. Small changes may have significant clinical impact if translated into the care of human infants at risk for ROP. To detect these potential small changes, a large number of retinas may be needed in each experiment to provide adequate statistical power. This potential need to use large numbers of retinas provides a rationale for the development of rapid scoring techniques such as counting clock hours in flatmounted ADPase-stained retina. We now report a direct comparison of scoring flatmounted ADPase-stained retinas with counting cell nuclei in an OIR rat model.
In addition, performing different experimental techniques on the same retina, such as ADPase staining and RNA extraction, is often not possible. There is a need to establish whether a sufficient similarity exists between left and right retinas of the same animal, so that paired retinas can be used for different techniques. Therefore, we also studied the intereye correlation of neovascularization score and retinal vascular area.

METHODS

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at our institution.

Animals and Experiment Environment

Seventy-five newborn Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN), were randomly assigned to three expanded litters ($n = 25$) as in our previous studies.5–7, 13 Pups were assigned to a single mother for the duration of the experiment. Mothers and litters received standard laboratory diet and water ad libitum. Light was cycled on a 12-hour-on, 12-hour-off, schedule, and the room was maintained at approximately $21^\circ$C. We used our published model of OIR5 with added inspired $10\%$ CO$_2$ (cycles of O$_2$, 80–10%) as the environment for this experiment. All 75 newborn rats were exposed to this environment for 7 days, followed by 5 days of room air recovery. Another three natural-sized litters (8–12 rats per litter) raised in room air were used as normal control animals.

All animals were killed on day 13 of life by a lethal intramuscular injection of ketamine and xylazine. Both eyes were enucleated and placed in 10% neutral buffered formalin for 90 minutes. The cornea, lens, and vitreous were surgically removed from each eye, and each retina was dissected.

Retinal Vascular Areas

All dissected retinas were placed in neutral buffered formalin overnight and processed for magnesium-activated ADPase staining as described by Lutty and McLeod.14 ADPase-stained retinas were temporarily flatmounted on microscope slides in phosphate buffered saline with a coverslip and photographed. The retinal images were captured by scanning the negatives with a flatbed scanner (ScanMaker III; Microtek Laboratory, Redondo Beach, CA). Vascularized and total retinal areas were calculated using commercial software (Analyze; Biomedical Imaging Resource, Mayo Foundation, Rochester, MN) by a masked examiner who traced the total and vascular retinal areas.

Counting Clock Hours of Neovascularization

Each retina was evaluated by three masked examiners for the presence and severity of neovascularization using light micro-

![Figure 1. ADPase-stained retina showing neovascularization.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933587/)
copy. The presence and severity of neovascularization were quantified in a manner that we and others have described previously (i.e., similar to a technique described for clinical ROP assessment). Briefly, each retinal quadrant was divided by visual estimation into three equal parts (clock hours; Fig. 1A), and each clock hour was scored for the presence or absence of abnormal neovascularization. The quadrants were created to be of approximately equal size, and each quadrant was defined by the cuts made at dissection. Any small discrepancies between quadrant sizes would be accounted for in the randomized design of previous and future studies with a large enough sample size. Preretinal neovascularization was defined as abnormal vascular structures morphologically distinct from the normal developing vasculature, including ridges, clumps, sheets, or tufts of endothelial cells (Figs. 1B, 1C). In this way, each retina was given a score between 0 and 12 clock hours by each examiner. The median value was assigned as the final grade for that retina. The total number of clock hours for each retina was considered to be an approximate measure of severity of neovascularization. A clock hour was scored as positive, regardless of the amount of neovascularization within that clock hour. Although this method yields an approximate score, a retina with 1 clock hour of neovascularization would clearly contain less neovascularization than a retina scored as 6 clock hours. We also included 18 control retinas from the room air-exposed litters to reduce the bias toward false positives, as we have described previously.

Counting Cell Nuclei
From the original 75 rats exposed to O₂ and CO₂, representative retinas that had been scored positive for neovascularization in OIR litters (n = 10), negative in OIR litters (n = 3), and room air control retinas (n = 3) were embedded in paraffin. OIR-positive retinas were intentionally selected to represent a range from the lowest score to the highest. After selection, all analyses were masked. Each entire peripheral retinal quadrant was serially sectioned at 6 μm and stained with hematoxylin and eosin (Fig. 2). Nuclei on the vitreal side of the internal limiting membrane were counted by a masked observer using light microscopy (magnification, ×100–250). This total number of nuclei counted per retina was defined as the nucleus count for further analysis. The preretinal neovascularization above the internal limiting membrane showed either luminal structures (Fig. 2B) or nuclei without a lumen (Fig. 2C). Because serial sections were counted, it is likely that some nuclei were counted twice. Although our nucleus count may overestimate the exact number of nuclei, this was a systematic error and ensured that no nucleus was missed.

Our method of counting cell nuclei is similar to that described by Smith et al. Nevertheless, our method differs from that of Smith et al. in that we serially sectioned each entire retinal quadrant and counted all sections. In these 16 sectioned retinas, the range of number of sections was 704 to 938 per retina; 12,900 sections were counted.

Statistical Analysis
Correlations between neovascularization grade and cell nucleus count and neovascularization and vascularized area for left and right eyes were evaluated using nonparametric Spearman rank coefficients. Nucleus counts in different groups were compared using an analysis of variance with Bonferroni adjustments for multiple t-tests. Interobserver agreement for incidence and severity of neovascularization was assessed using the κ statistic and by calculating the percentage of retinas in which every observer’s score was within 1 clock hour of the median.

RESULTS
Comparison of Counting Clock Hours with Counting Cell Nuclei
In 16 serially sectioned paraffin-embedded retinas, the nucleus count above the internal limiting membrane was 0 to 44 in room air control retinas (Fig. 3A), 0 to 40 in OIR-negative
retinas, and 250 to 5634 in OIR-positive retinas (Fig. 3A). The OIR-positive retinas had higher median nucleus counts than the OIR-negative and room air control retinas (Fig. 3A). The nucleus counts were highly correlated with the clock hour scores ($r_s = 0.95, P = 0.0001$, Fig. 3B).

Comparison of the Vasculature of Right to Left Eyes

Similar to our previous studies of OIR, not all rats survived. Specifically, 39 of 75 rats completed the study resulting in 78 OIR retinas for analysis. All 78 OIR ADPase-stained retinas and 18 room air control retinas (total 96) were technically readable and could be scored. Of the 78 OIR retinas, preretinal neovascularization occurred in 69% of the right eyes versus 72% in left eyes ($P = 0.8$). None of room air control retinas was scored positive for preretinal neovascularization. The clock hour scores in the OIR retinas ranged from 0 to 12 in the left eyes and from 0 to 9 in the right eyes. The median of severity was 3 clock hours in both left and right eyes. Data from the 78 OIR retinas (39 pairs) were then used in the following paired comparisons. There was a significant correlation in the severity of neovascularization between right and left eyes ($r_s = 0.76, P = 0.0001$, Fig. 3C), and the ratio of retinal vascular area to total retinal area ($r_s = 0.81, P = 0.0001$, Fig. 3D).

Interobserver Agreement for Neovascularization Scores

For the 96 scored retinas (78 OIR and 18 control retinas), interobserver reliability among three examiners was evaluated using the $\kappa$ statistic (Table 1). These values ($\kappa = 0.68 - 0.78$) indicate the interobserver agreement for the incidence of neovascularization was good to excellent. The individual grade for severity of neovascularization among the three examiners was within 1 clock hour of the median in 81 of the 96 retinas (84%).

**DISCUSSION**

In the present study, we validated the method of counting clock hours of neovascularization in flatmounted ADPase-stained rat retinas by serially sectioning all four quadrants of each retina and counting the nuclei structurally adjacent to the vitreal side of the internal limiting membrane. Our results confirmed that the assigned scores for neovascularization in the flatmounted ADPase-stained retinas were highly correlated with the nucleus count. We also found that the incidence and severity of neovascularization, as well as vascular areas, were highly correlated between the right and left eyes.

Counting clock hours in flatmounted ADPase-stained retina has many advantages over other previously described methods of quantifying neovascularization in the animal models of ROP. The technique is rapid and allows scoring of many more retinas in a shorter time. In our experience, it takes 2 working days to quantify neovascularization in a single retina using the

**Table 1. Interobserver Agreement for the Incidence of Neovascularization**

<table>
<thead>
<tr>
<th>Examiners</th>
<th>$\kappa$</th>
<th>95% Confidence Interval</th>
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<tbody>
<tr>
<td>A vs B</td>
<td>0.68</td>
<td>0.53--0.83</td>
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<tr>
<td>A vs C</td>
<td>0.72</td>
<td>0.53--0.86</td>
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<tr>
<td>B vs C</td>
<td>0.78</td>
<td>0.66--0.91</td>
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nucleus-counting technique. However, during the same 2-day period, we can process and grade approximately 80 retinas by counting clock hours in flat-mounted ADPase-stained specimens. Counting clock hours in flat-mounted ADPase-stained retina is also a reliable method for quantifying neovascularization in the rat model of ROP. In our present study, the ADPase-stained OIR retinas scored negative for neovascularization (0 clock hours) had a maximum nucleus count of 40, which was similar to room air control retinas (44 nuclei). This low score in negative retinas contrasted with 250 to 5634 nuclei in retinas scored positive for neovascularization. We are therefore confident that the method of counting clock hours in flat-mounted ADPase-stained rat retinas provides a reliable assessment of neovascularization in the rat model of ROP. The morphology of neovascularization found in other rat models of ROP is very similar to that in our model.\(^1\) We therefore believe that the technique of counting clock hours in flat-mounted ADPase-stained retinas also can be applied to these similar rat models, regardless of litter size or oxygen exposure.

Clinical data have shown that infants with ROP had a similar ROP grade between left and right eyes.\(^1\) In the present study, we confirmed that there was a significant correlation in the ratio of retinal vascular area to total retinal area between left and right eyes, and there was also a significant correlation in the severity of neovascularization (clock hour score) between left and right eyes. Despite the variability among the animals, the responses between the paired eyes were remarkably similar in both vascularized area and the severity of neovascularization. Our results suggest that it is possible to use one eye for assessment of vascular morphology and the fellow eye for other studies (e.g., growth factor analysis).

Regarding the limitations of grading neovascularization by counting clock hours, it is possible that the technique may have variability among different examiners, partly due to the visual estimation of each clock hour border. Nevertheless, in the present study, we found excellent agreement between the individual grades for each retina with the final grade of the retina among three examiners. The individual grades for neovascularization from three examiners were within 1 clock hour of the median grade in 81 of 96 retinas (84%). In a further analysis of agreement, the \(\kappa\) statistic indicated the interobserver agreement for presence of neovascularization was good to excellent (Table 1). Another limitation of our study is that a ridge of neovascularization (Fig. 1B) may obtain the same score as several tufts (Fig. 1C). Such a clock hour is scored as positive, regardless of the amount of neovascularization in that clock hour. Nevertheless, these potential problems did not adversely influence the correlation of clock hour score to nucleus count (Fig. 3B). In addition, we expect that such differences would be accounted for, over a large number of retinas, in an experimental randomized study.

Our results confirmed that counting clock hours in ADPase-stained retinas is a valid and reliable method of grading neovascularization in the rat model of ROP. The method has the advantage of being less time-consuming and labor-intensive than counting cell nuclei. Incidence of neovascularization, severity of neovascularization, and vascularized areas were similar between left and right eyes, and this may permit the use of paired eyes for complementary research techniques.

References