Project Overview

Purpose

This research serves as a preclinical evaluation of extra virgin olive oil (EVOO) and lutein-in-extra-virgin-olive-oil (LEVOO) as a new avenue for the prevention of retinopathy of prematurity. Retinopathy of prematurity is a multifactorial retinal disorder that is a major cause of preventable childhood blindness among preterm infants who receive supplemental oxygen therapy. Since current treatment methods are often invasive and require long-term monitoring, the non-invasive strategy of using lutein supplementation to reduce abnormal neovascular area and thus improve the outcome of retinopathy of prematurity is investigated. The two major aims of this internship are to 1) learn about different laboratory techniques needed for conducting research and 2) to investigate microglial activation in EVOO and LEVOO treated oxygen-induced-retinopathy pups.

Hypothesis

Lutein is a natural xanthophyll carotenoid with strong antioxidant properties and is generally regarded as safe. Previous studies have shown that daily intraperitoneal lutein injection promoted normal retinal vascular growth. Extra virgin olive oil is also globally appreciated as healthy with its antioxidizing and immune supportive properties. Its high oleic acid content supports lutein absorption and increases its bioavailability. Thus, it is hypothesized that EVOO and LEVOO are safe and effective nutrient supplementations for mouse neonates and can protect their retinae from oxygen-induced retinopathy.

Methodology

Oxygen-induced retinopathy (OIR) is a well-recognised animal model for mimicking retinopathy of prematurity in humans. Postnatal day 7 mouse neonates are exposed to 75% oxygen before being returned to room air to maximize vaso-obliteration on postnatal day 12. EVOO and LEVOO with the dosage of 3.6ug/g are administered daily by intraperitoneal injection from postnatal day 12 onwards. Retinal vascularization peaks on postnatal day 17 which is when the mouse neonates are sacrificed. Retinal paraffin sections and flat mounts are then prepared for morphological examination. Microglial distribution that reflects inflammatory response is a major focus during this internship while other investigations involving vasculature, cytokines, oxidative stress and lipid metabolism are also examined throughout the course of this research.

Procedures

Retinal microglia are detected by staining with an antibody against Iba-1, a well characterized retinal microglia marker. Cluster of differentiation 68 (CD68) immunohistochemistry is also used to identify activated microglia which may indicate inflammation. The selection of fields of view in avascular area (AA), mid-peripheral area without neovascularization (non-tufts) and neovascularized areas (NV) are randomised. Confocal images of central and mid-peripheral zones from superficial and deep retinal vascular layers are then collected using a confocal laser scanning microscope. The criteria for counting cell bodies include that their shapes must be round or hollow in the middle and of at least one cell size. Round shapes formed by cell tentacles are ignored. The number of cells stained red are regarded as the total number of microglia (Figure 1) and the number of cells co-stained with red and yellow or teal color are regarded as activated microglia (Figure 2). Counting is performed twice on different days and the average of the two results is taken.

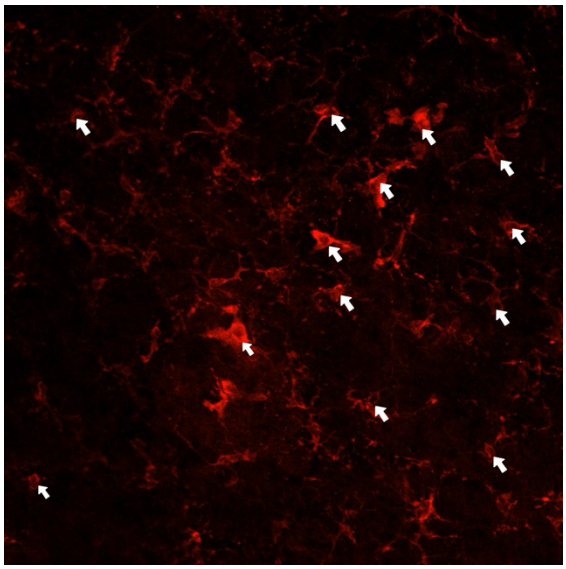

Figure 1: Example of microglial cell bodies stained by Iba1.

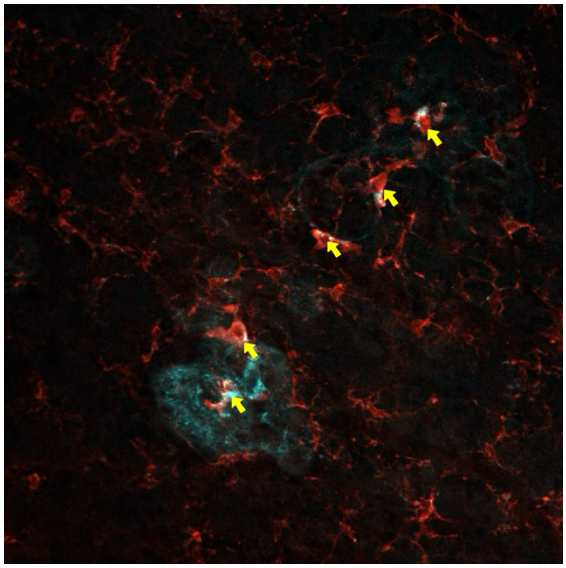

Figure 2: Example of microglial cell bodies co-stained with Iba1 and CD68.

Results

A total of 74 images were counted and analyzed with 2 or 3 images taken from the avascular area, 3 or 4 images taken from the Non-Tufts areas and 4 images taken from the NV areas from each retinal sample. Out of the 7 mice used, 3 were treated with EVOO while 4 were treated with LEVOO. The difference between microglial activation percentage of the EVOO and LEVOO groups is not statistically significant. For samples obtained from the AA area, the activation percentage of the LEVOO group (45%) is slightly lower than that of the EVOO group (50%). This difference is also seen in the Non-Tufts areas where the activation percentage of LEVOO group (52%) is lower than that of the EVOO group (54%). However, the activation percentage of the LEVOO group (75%) in the NV area is higher than that of the EVOO group (67%). (Figure 3) The total number of microglial cells and activated microglia per mm^2 of the LEVOO group is also slightly larger than that of the EVOO group. (Figure 4) A probable reason for the lack of statistical significance despite the slight differences is sample size being investigated is too small.

Besides the quantification of microglial cells, various laboratory work is also performed. Retinal flat mount is a valuable micromanipulation skill acquired during this internship. (Figure 5) The technique needed to dissect and manipulate a small structure under the microscope requires

patience and practice and is a beneficial skill to obtain for a future medical career. Immunohistochemistry staining is also attempted for the visualization of different retinal cell layers. (Figure 6) Understanding of the steps such as antigen retrieval, addition of primary and secondary antibodies as well as fluorescent examination and photo-taking are also essential takeaways from this internship. Preparation and staining samples with hematoxylin and eosin are also performed. (Figure 7) Observing other laboratory work such as electroretinography, western blot and cell line transfer are also learning experiences that help develop a deeper understanding on how scientific research is conducted. Additionally, attending other events such as the Ophthalmology Grand Round, Distinguished Lecture and lab meetings are also valuable experiences for exposure to research-related academic exchanges.

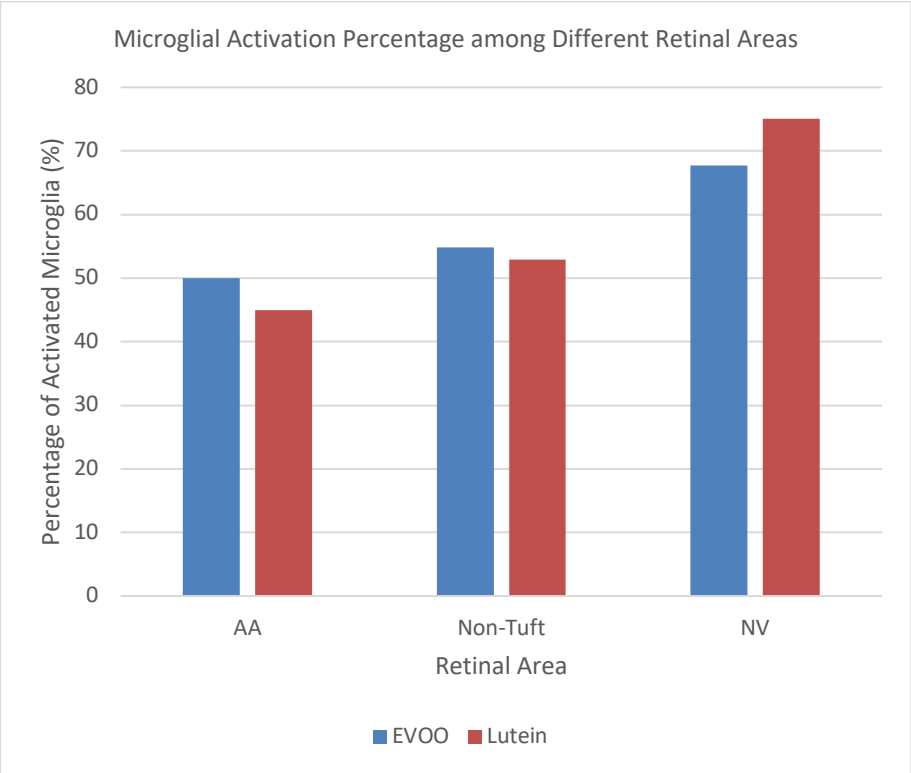

Figure 3: Bar chart showing microglial activation differences.

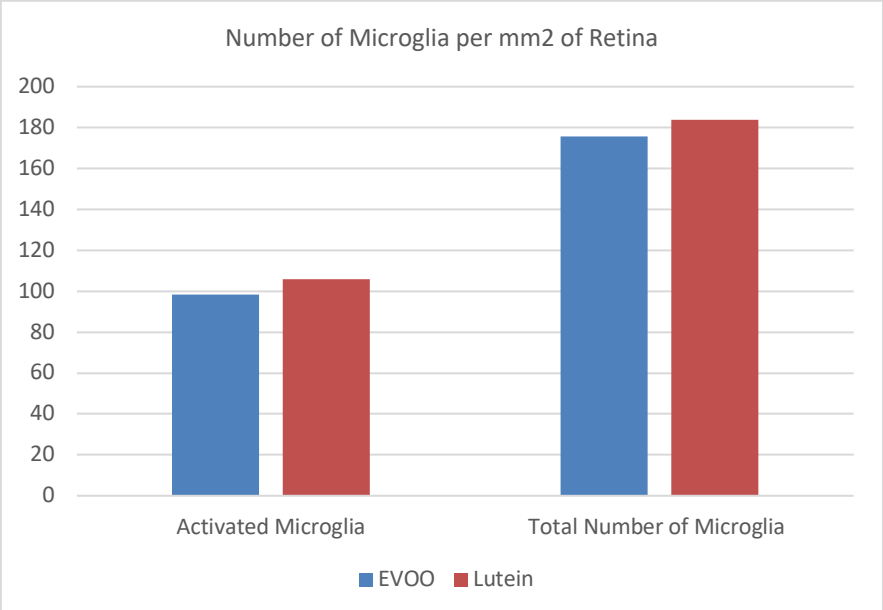

Figure 4: Bar chart showing number of cells per mm^2 of retina.

	First Count				Second Count				Averaged Data				
	Iba1+	Iba1+ CD68+	Percentage of Microglia Activated	Activation % (avg of each area)	Iba1+	Iba1+ CD68+	Percentage of Microglia Activated	Activation % (avg of each area)	microglia mean	microglia/ mm2	activated no	activated/ mm2	activation %
EVOO 3-02F AA1	25	12	48.00%		25	12	48.00%						
EVOO 3-02F AA2	18	10	55.56%	51.78%	21	11	52.38%	50.19%	22.25	217.29	11.25	109.86	50.56%
EVOO 3-02F Non-Tuft 1	6	1	16.67%		6	1	16.67%						
EVOO 3-02F Non-Tuft 2	8	3	37.50%		8	2	25.00%						
EVOO 3-02F Non-Tuft 3	5	3	60.00%	38.06%	4	3	75.00%	38.89%	6.17	60.22	2.17	21.16	35.14%
EVOO 3-02F NV1	19	13	68.42%		22	15	68.18%						
EVOO 3-02F NV2	17	9	52.94%		15	10	66.67%						
EVOO 3-02F NV3	11	6	54.55%		10	6	60.00%						
EVOO 3-02F NV4	19	11	57.89%	58.45%	22	15	68.18%	64.95%	15.67	152.99	9.83	96.03	62.77%
EVOO 4-00F AA1	28	16	57.14%		25	14	56.00%						
EVOO 4-00F AA2	22	13	59.09%		17	9	52.94%						
EVOO 4-00F AA3	34	19	55.88%	57.37%	29	18	62.07%	57.00%	25.83	252.28	14.83	144.86	57.42%
EVOO 4-00F Non-Tuft 1	17	14	82.35%		16	13	81.25%						
EVOO 4-00F Non-Tuft 2	26	14	53.85%		24	12	50.00%						
EVOO 4-00F Non-Tuft 3	19	13	68.42%		15	10	66.67%						
EVOO 4-00F Non-Tuft 4	14	13	92.86%	74.37%	13	11	84.62%	70.63%	18.00	175.78	12.50	122.07	69.44%
EVOO 4-00F NV 1	20	14	70.00%		14	12	85.71%						
EVOO 4-00F NV 2	25	19	76.00%		23	15	65.22%						
EVOO 4-00F NV 3	19	12	63.16%		14	9	64.29%						
EVOO 4-00F NV 4	17	10	58.82%	67.00%	14	8	57.14%	68.09%	18.25	178.22	12.38	120.85	67.81%
EVOO 5-10M AA1	38	18	47.37%		20	10	50.00%						
EVOO 5-10M AA2	21	9	42.86%		13	5	38.46%						
EVOO 5-10M AA3	22	8	36.36%	42.20%	12	3	25.00%	37.82%	21.00	205.08	8.83	86.26	42.06%
EVOO 5-10M Non-Tuft 1	18	9	50.00%		15	6	40.00%						
EVOO 5-10M Non-Tuft 2	14	8	57.14%		9	6	66.67%						
EVOO 5-10M Non-Tuft 3	10	6	60.00%		11	6	54.55%						
EVOO 5-10M Non-Tuft 4	20	15	75.00%	60.54%	20	14	70.00%	57.80%	14.63	142.82	8.75	85.45	59.83%
EVOO 5-10M NV 1	24	15	62.50%		23	15	65.22%						
EVOO 5-10M NV 2	25	18	72.00%		22	16	72.73%						
EVOO 5-10M NV 3	18	13	72.22%		13	10	76.92%						
EVOO 5-10M NV 4	19	16	84.21%	72.73%	17	14	82.35%	74.31%	20.13	196.53	14.63	142.82	72.67%
L36 3-10F AA 1	45	20	44.44%		39	17	43.59%						
L36 3-10F AA 2	41	17	41.46%	42.95%	41	18	43.90%	43.75%	41.50	405.27	18.00	175.78	43.37%
L36 3-10F Non-Tuft 1	11	4	36.36%		14	7	50.00%						
L36 3-10F Non-Tuft 2	10	6	60.00%		11	7	63.64%						
L36 3-10F Non-Tuft 3	11	8	72.73%		15	11	73.33%						
L36 3-10F Non-Tuft 4	10	5	50.00%	54.77%	12	6	50.00%	59.24%	11.75	114.75	6.75	65.92	57.45%
L36 3-10F NV 1	14	11	78.57%		15	11	73.33%						
L36 3-10F NV 2	14	9	64.29%		12	8	66.67%						
L36 3-10F NV3	15	10	66.67%		15	10	66.67%						
L36 3-10F NV4	16	12	75.00%	71.13%	17	13	76.47%	70.78%	14.75	144.04	10.50	102.54	71.19%
L36 3-20F AA 1	14	11	78.57%		12	8	66.67%						
L36 3-20F AA 2	15	5	33.33%		15	7	46.67%						
L36 3-20F AA 3	19	12	63.16%	58.35%	16	10	62.50%	58.61%	15.17	148.11	8.83	86.26	58.24%
L36 3-20F Non-Tuft 1	13	11	84.62%		12	10	83.33%						
L36 3-20F Non-Tuft 2	10	9	90.00%		11	9	81.82%						
L36 3-20F Non-Tuft 3	16	7	43.75%		16	7	43.75%						
L36 3-20F Non-Tuft 4	12	7	58.33%	69.17%	14	8	57.14%	66.51%	13.00	126.95	8.50	83.01	65.38%
L36 3-20F NV 1	18	16	88.89%		22	18	81.82%						
L36 3-20F NV 2	16	14	87.50%		19	16	84.21%						
L36 3-20F NV 3	22	17	77.27%		25	16	64.00%						
L36 3-20F NV 4	16	15	93.75%	86.85%	16	13	81.25%	77.82%	19.25	187.99	15.63	152.59	81.17%
L36 4-01F AA 1	13	6	46.15%		14	6	42.86%						
L36 4-01F AA 2	17	11	64.71%		14	8	57.14%						
L36 4-01F AA 3	19	9	47.37%	52.74%	17	8	47.06%	49.02%	15.67	152.99	8.00	78.13	51.06%
L36 4-01F Non-Tuft 1	16	8	50.00%		19	10	52.63%						
L36 4-01F Non-Tuft 2	13	8	61.54%		11	5	45.45%						
L36 4-01F Non-Tuft 3	16	8	50.00%		12	5	41.67%						
L36 4-01F Non-Tuft 4	16	13	81.25%	60.70%	12	7	58.33%	49.52%	14.38	140.38	8.00	78.13	55.65%
L36 4-01F NV 1	32	20	62.50%		29	17	58.62%						
L36 4-01F NV 2	32	26	81.25%		27	20	74.07%						
L36 4-01F NV 3	41	30	73.17%		34	24	70.59%						
L36 4-01F NV 4	33	29	87.88%	76.20%	26	23	88.46%	72.94%	31.75	310.06	23.63	230.71	74.41%
L36 5-00M AA 1	12	2	16.67%		11	1	9.09%						
L36 5-00M AA 2	24	5	20.83%		20	5	25.00%						
L36 5-00M AA 3	14	7	50.00%	29.17%	15	6	40.00%	24.70%	16.00	156.25	4.33	42.32	27.08%
L36 5-00M Non-Tuft 1	16	7	43.75%		16	6	37.50%						
L36 5-00M Non-Tuft 2	18	5	27.78%		15	4	26.67%						
L36 5-00M Non-Tuft 3	16	5	31.25%		14	4	28.57%						
L36 5-00M Non-Tuft 4	12	5	41.67%	36.11%	10	3	30.00%	30.68%	14.63	142.82	4.88	47.61	33.33%
L36 5-00M NV 1	11	5	45.45%		14	5	35.71%						
L36 5-00M NV 2	21	16	76.19%		17	13	76.47%						
L36 5-00M NV 3	18	16	88.89%		20	15	75.00%						
L36 5-00M NV 4	23	19	82.61%	73.29%	19	16	84.21%	67.85%	17.88	174.56	13.13	128.17	73.43%

Figure 5: Raw data showing the quantification of microglia and activated microglia.

Figure 6: Retinal Flat Mount.

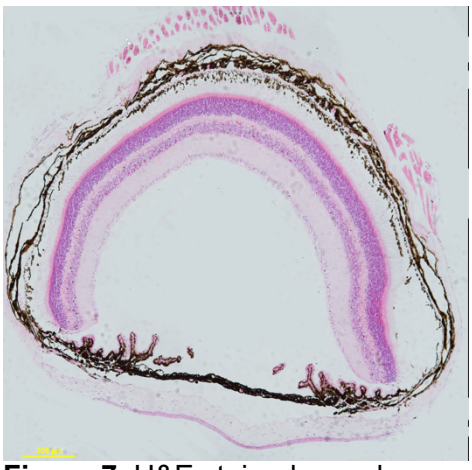

Figure 7: H&E stained sample.

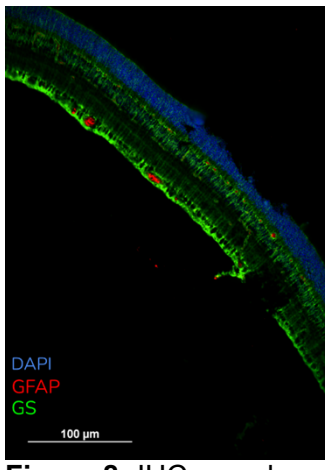

Figure 8: IHC sample.

Difficulties, Resolvement and Improvement

The major difficulty faced during the research is the subjective classification of cell bodies. As some of the tentacles formed oval or round shapes that may be easily confused with the circular morphology of the cell bodies, it is difficult at times to determine whether the shape should be regarded as a microglial cell. To increase the reliability of the data obtained, repeated counting is performed, and the margin of acceptable error is set at 20%. Moreover, examples of which structures should be regarded as cell bodies while other structures that should be disregarded are recorded for future reference to ensure consistency. To further improve the accuracy of this experiment, another member of the research team may again perform counting to further minimize potential human errors. The experiment can also be repeated with a larger sample size to increase the chances of obtaining stastically significant results.

Impact of the Research and Reflections

Publication of research data in journals related to vision studies is anticipated. Meanwhile, the study helps to assess the potential translational value of lutein-in-extra-virgin-olive-oil for better neonatal and pediatric care in Hong Kong by preventing or improving the outcome of retinopathy of prematurity.

This internship has been an invaluable experience to gain exposure and deepen my understanding of academic research. Not only has these six weeks further increased my interest in ophthalmology, I also understood the potential benefits that evidence-based research can bring to the wider community. I learnt to appreciate the patience and passion needed for conducting impactful research. By actively exploring various research work and solving problems along the way, I cultivated essential critical thinking and time management skills that are essential for tackling other challenges in the future. This attachment has surely been a memorable and impactful experience for me, and I look forward to continuing my journey as a Laidlaw Scholar in the future.