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Systemic Fat Embolism-Induced Accumulation of Fat Droplets in the Rat Retina

Research Article

Caroline G. Olson[1], Landon J. Rohowetz[1], A. Paula Monaghan-Nichols[2], Alan Poisner[3], Agostino Molteni[4], Peter Koulen[1,2,5*]

[1] Vision Research Center, University of Missouri – Kansas City, School of Medicine, Kansas City, 7 Missouri, USA.
[2] Department of Biomedical Sciences, University of Missouri – Kansas City, School of Medicine, Kansas City, Missouri, USA.
[3] University of Kansas Medical Center, Kansas City, Kansas, USA.
[4] Department of Pathology, University of Missouri- Kansas City, School of Medicine, Kansas City, Missouri, USA.
[5] Vision Research Center, University of Missouri – Kansas City School of Medicine, Department of Ophthalmology, Kansas City, Missouri USA.

Abstract

Fat embolism syndrome has been implicated in damage to the blood-retina barrier and retinopathies, thereby representing a potential target for therapy development. In order to characterize features of retinal emboli including size, number, and location, an established rat model of pulmonary fat embolism was employed. Adult rats received caudal vein injections of triolein and the short term (2 days) and long term (70 days) consequences of systemic fat emboli were examined histologically in the retina. Significant numbers and distribution of emboli were observed in the retina at 2 days but the percent and location of emboli in the retina from experimental animals at 70 days was not significantly different from controls. We conclude that systemic fat embolism can cause fat droplet emboli in the retina for brief periods of time, but that they resolve with time.

Keywords: Triolein; Fat Embolism Syndrome; Retina; Emboli; Traumatic Injury.

Introduction

Systemic fat embolism induced by intravenous injection of triolein results in the accumulation of fat droplets in pulmonary tissue leading to septal and arterial inflammation and eventually pulmonary fibrosis in rats.[1] A cat model of fat emboli led to a significant disruption of the blood-retina barrier.[2] Clinical observations indicate a potential involvement of fat embolisms in retinopathy [3-7], presenting a potential target for therapy development.[8] This is of particular significance because retinopathy is a leading cause of irreversible blindness in people over 50 years of age in the developed world.[9, 10] Retinopathies are separated into non-neovascular (dry, atrophic, or nonexudative) and neovascular (wet, or exudative) forms. While neovascular retinopathy represents only about 10-15% of cases, it constitutes over 80% of severe vision loss associated with retina degeneration. [9, 10] Numerous causes of pathogenesis have been postulated including ischemia.[10] Several studies have linked embolisms and micro-embolisms and subsequent cellular signaling pathways with ischemic disease.[2-7, 9, 10] In patients with fat embolism syndrome (FES), fifty percent experienced neovascular retinopathy. Retinopathy seen in FES resembles diabetic retinopathy and includes macular edema, cotton wool spots, and bilateral 48 intraretinal hemorrhages.[11, 12] While fat emboli are known to cause relatively less damage than other types of emboli, the inflammatory effect along with the mechanical damage and local ischemia can cause lasting damage.[2] This ischemia can trigger release of VEGF leading to activation of an angiogenic cascade with neovascularization and subsequent intraretinal hemorrhages. These hemorrhages can obscure vision and cause macular edema. The most common lasting visual deficits are paracentral visual scotomas which can negatively impact patient's activities of daily living and quality of life.[12] This study utilizes a rat model of fat embolism syndrome utilizing triolein injection at two time points time from injection to sacrifice to examine the characteristics of the emboli including size, number, and location of emboli in the retina. Acute (2 days) and long term (70 days) consequences of triolein injection were examined in order to better understand the timeline of the disease.

***Corresponding Author**:
Peter Koulen,
Vision Research Center, University of Missouri – Kansas City School of Medicine, Department of Ophthalmology, Kansas City, Missouri USA.
Tel: 816-235-6733
E-mail: koulenp@umkc.edu

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Material and Methods

This study was conducted under the approval of the University of Missouri at Kansas City Institutional Animal Care and Use Committee (IACUC). Animal care and procedures were in accordance with institutional guidelines. All rats were given ad libitum access to food and water, and observed at various time points after the injections. No animals died before euthanasia using inhalation isoflurane (Sigma Corp., St. Louis, MO).

The histology material was prepared from a total of 19 male Sprague- Dawley rats (Harlan Laboratories, Indianapolis, IN; body weight, approximately 300g) divided into 2 cohorts: Rats received an intravenous tail vein injection of either 0.2ml of sterile physiological saline or 0.2ml pure triolein (glyceryl trioleate, Sigma grade; Sigma Corp., St. Louis, MO) and were euthanized 2 days (cohort 1) or 70 days later (cohort 2).

Following euthanasia, the bilateral enucleated eyes and optic nerves were either snap frozen for Oil red O staining of fat emboli or fixed in formaldehyde overnight and processed through a sucrose gradient for frozen sections.

The specimens were embedded in optimal cutting temperature (OCT) glue using and cryostat-sectioned at 14-micrometer sections on gelatin-coated slides. Each slide contained one sample from the control group and one experimental. Slides were stained using Hematoxylin and Eosin (Technovit H7100/H8100, Electron Microscopy Services, Hatfield, CT) and Oil Red O 77 (Solvent Red 27, Sudan Red 5B; Oil Red O in 0.5% solution in propylene glycol, Poly Scientific R&D corps, Bay Shore, NY) stains to identify fat emboli. Fat emboli were identified and imaged. Images were taken of H&E stained and Oil Red O stains at 10x and 20x magnification using color microscope (Axiovert 40CFL, Zeiss, Gottingen, Germany) and Leica software (Leica Microsystems Inc., Buffalo Grove, IL). Analysis using Image-J/FIJI software (Image J, National Institute of Health, Bethesda, MD) was performed to quantify the location, thickness, and size of emboli.

In statistical analysis, chi-square tests were used in intergroup comparisons of categorical variables. In comparisons between intervention and cohort, ANOVA was used to compare size of 86 emboli between cohorts and emboli area per retinal area between cohorts. P values lower than 0.05 were considered statistically significant. The analyses reported were performed using GraphPad Prism Version 8 (GraphPad Software, San Diego, CA), Microsoft Excel (Microsoft Corporation, Redmond, WA), and IBM SPSS Statistics Version 25 (IBM Corp., Armonk, NY).

Results

Sixteen adult rats were distributed into two cohorts and injected with saline or Triolein. In the acute treatment group, cohort 1, 4 rats received saline treatment and 4 received triolein treatment. 2 days later, eyes were removed and one eyecup per rat was processed for immunohistochemical staining. In cohort 2, the long term treatment group, 4 rats received either saline or triolein and were sacrificed at 70 days. Samples were fixed, sectioned and subsequently analyzed from multiple anatomically-separate regions of the retina. Two sections were excluded from analysis due to overstaining. Twenty-two emboli were found in samples from cohort 1 while 2 emboli were found in samples from cohort 2.

Analysis was completed with each rat as an individual unit of measure, as well as each slide as a unit of measure. Table 1 lists the overall incidence of retinal embolization between all saline treated and triolein treated rats using analysis of each slide (Pearson chi-square: 4.173; P = 102 0.243). Triolein-treated rats in cohort 1 exhibited the greatest incidence of retinal embolization at 23.5% compared to saline-treated rats at 5.56% (Table 1). Oil red O stains fat and will stain not only for Triolein but will also detect endogenous lipid content in the normal eye leading to occasional Oil Red O positivity. In just examining cohort 1 comparing saline and triolein, several of the triolein-treated rats in cohort 1 exhibited multiple emboli (up to 14 distributed throughout the retina). Analysis of each rat as an individual also shows similar rates of embolization, with the highest rate (50%) in cohort 1 triolein injected (Chi square of 0.873 (significance 0.832)). Using Levene's test for equality of variances, there were significantly more emboli in the triolein 110 treatment group of cohort 1 (P = 0.008) (Tables 1, 2).

To determine the effect of time on fat embolism, cohort 2 eyes were examined 70 days after triolein or saline injection. Both triolein-treated and saline-treated rats in cohort 2 had significantly lower numbers of emboli than in cohort 1, suggesting that there was clearance of the emboli over the 9.5-week span separating the two groups. The strength of association between triolein administration and embolus development based on being in either cohort 1 and 2 was very strong based on odds ratio of 0.217 (95% CI 0.021 to 2.191).

Size of emboli comparing all rats in cohort 1 and cohort 2 using analysis by slide was not significant based on ANOVA of 0.840 (P = 0.477). Size of emboli in cohort 1 only comparing triolein-

Table 1: Incidence of retinal embolization between each condition as analyzed by slide.

Condition	Emboli absent	Emboli present
Cohort 1 Saline	16 (94.1%)	1 (5.88%)
Cohort 1 Triolein	13 (76.4%)	4 (23.5%)
Cohort 2 Saline	15 (93.8%)	1 (6.25%)
Cohort 2 Triolein	15 (93.8%)	1 (6.25%)

(Pearson chi-square: 6.371, P = 0.095). Triolein-treated rats in cohort 1 exhibited the greatest incidence of retinal embolization at 23.5% while saline-treated rats in cohort 1 demonstrated the lowest incidence at 5.56%. Cohort 2 overall had lower rates of embolization than cohort 1.Endogenous lipid stained in the saline treatment groups.

treated versus saline-treated rats showed that the average embolus diameter was not significantly greater in the saline-treated group (P = 0.579). Analysis of cohort 1 saline and triolein treated rats using each rat as an individual showed that the average embolus diameter was not significantly different (P = 0.894) (Table 3).

In the triolein treated rats, there was a wide variety in size of emboli. In the saline treated rats, there were very few areas that stained with Oil Red O, so the larger areas of endogenous lipid may have appeared significant.

In both cohorts, it is expected that there would be no difference between retinal length. This was included to facilitate the data of emboli per length. Retinal thickness was also expected to be not significantly different.

No statistically significant difference was found regarding the location of emboli in the retina as seen in Figure 1.

Discussion

Fat embolism syndrome (FES) occurs when fat emboli are mobilized in the circulation, most commonly after fracture of long bones, but also in pancreatitis, cesarean section, sickle cell bone crisis, liposuction, and bone marrow transplant.[1, 2, 13-15] It is thought that up to 35% of patients may have FES after traumatic injury.[16] The mechanism behind fat release after trauma is thought to be either from elevated intramedullary pressure fol-

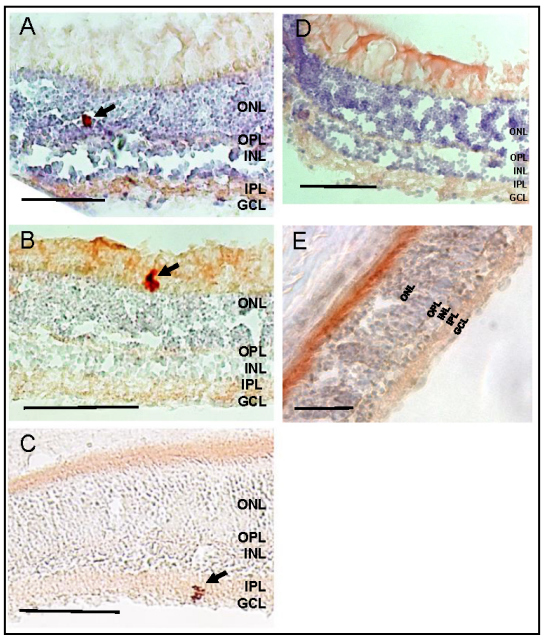
Table 2. Chi-square analysis between cohorts in saline and triolein as seen in table 1 for incidence of retinal embolization.

	Value	Asymptotic Significance (2-sided)
Pearson Chi-Square	4.173[a]	0.243
Likelihood Ratio	3.628	0.305
Linear-by-Linear Association	0.16	0.689
Number of total samples	67	

Table 3. Independent-samples t-test comparing cohort 1 with saline and cohort 1 with triolein using each slide as a unit.

	Sig, (2-tailed)	Cohort	Mean	Std Deviation	Std Error of the Mean
Number of Emboli	0.152	Cohort 1 saline	0.06	0.209	0.056
		Cohort 1 triolein	0.41	2.92	0.243
Average Embolus Diameter	0.579	Cohort 1 saline	3.39	12.75	3.4
		Cohort 1 triolein	5.95	12.75	3
Total Embolus Diameter	0.336	Cohort 1 saline	3.4	10.69	3.4
		Cohort 1 triolein	13.48	41.28	10.01
Retinal Thickness	0.07	Cohort 1 saline	220.8	38.94	9.18
		Cohort 1 triolein	247.3	44.63	10.83
Number of Emboli Per Thickness	0.132	Cohort 1 saline	0.000	0.001	0.000
		Cohort 1 triolein	0.004	0.004	0.001
Distance From Edge (RGC Side)	0.23	Cohort 1 saline	47.27	N/A	N/A
		Cohort 1 triolein	129.9	50.74	25.37
Percent Distance From Edge	0.174	Cohort 1 saline	20.6	N/A	N/A
		Cohort 1 triolein	56.4	20.48	10.24

Figure 1. Oil Red O staining of fat emboli in the retina. Arrows indicate fat emboli.



A. Fat embolus in the outer nuclear layer of the retina in triolein treated Cohort 1 (2 days) tissue. B. Embolus in the photoreceptor layer of triolein treated Cohort 1 (2 days) tissue. C. Embolus in the inner plexiform layer of triolein treated Cohort 2 (70 days) tissue. D. Saline treated control from cohort 1 (2 days). E. Saline treated control from cohort 2 (70 days). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 50μm.

lowing mechanical force causing direct release of fat from bone marrow, or systemic inflammation leading to mobilization of free fatty acids from bone marrow.[13] Fat emboli are known to cause local inflammation and increased vessel permeability.[13] Depending on the size of the embolism and its entry point into the circulation, emboli can cause symptoms in tissues throughout the body commonly including the lungs, brain, skin, and eyes. In FES, 50-60% of patients can have ocular findings on exam including cotton wool spots and retinal hemorrhages.[2] Delayed symptom onset is typical, with symptoms often occurring 24-48h after injury, which has been postulated to be due to lipolysis of emboli or delayed release of fat droplets from bone.[1, 2] Retinopathy caused by FES typically resolves over the course of months, but visual scotomas can persist due to changes in the retinal pigment epithelium and optic nerve atrophy.[6]

Utilizing a rat model to induce retinal emboli allows us to characterize these emboli and better understand the timeline of the disease. Previous studies have examined the effect of fat emboli on the blood-retina barrier but have not yet characterized the effects of altering parameters of the triolein injection. This study examined the fat emboli that occur in the retina after injection of triolein fat at varying times. The results of this study show that the shortest interval between injection and sacrifice had the most emboli present. Cohort 2 which had a longer time interval (70 days) had significantly fewer to no emboli. This shows that fat emboli may only remain in the retina for a short period of time before resolving but that long term damage can occur as a secondary consequence of the fat emboli. In this study, the average embolus diameter was 0.85μm, which is far below the average diameter of retinal blood vessels (45μm). Large fat droplets typically get stuck in the pulmonary vessels, but droplets that are small enough to make it to the retina may be easily cleared.6 The cohort with the larger dose of triolein may have had more fat emboli in their lungs and larger vessels instead of small droplets that could make it to the retina. Due to the clinical vision symptoms of fat embolism to the retina, it is clear that these emboli, although small and transient, create lasting damage.

The mechanisms behind the damage caused by these small emboli are thought to be threefold. First, there is endothelial damage from toxic free fatty acid release, second, there is mechanical endothelial damage, and third, there is capillary obstruction leading to ischemia.[2, 17] Toxic damage to endothelium is primarily due to the hydrolysis of fat by triglyceride lipases to yield oleic acid.1 Mechanical damage to the vascular endothelium caused by these small emboli has been shown to increase the permeability of the blood retina barrier which could lead to long term damage in the retina.[2] Capillary obstruction causes short term hypoxia which can present with ischemic changes on ocular exam including cotton-wool spots, hemorrhages, and retinal edema.[17] Emboli both in tissue and in vasculature can cause an inflammatory reaction which can cause local damage and long term deficiencies.

Resolution of emboli may happen through a cellular mechanism. It has been proposed that fat droplets can be phagocytosed by macrophages which then can stimulate an inflammatory response.[1] Macrophages can engulf fat emboli and metabolize them into oleic acid while also stimulating mast cells to activate the renin-angiotensin-aldosterone system.[1] It has been shown that fat emboli in the lungs can cause pulmonary edema through this mechanism.1 Promising animal studies in the lung have shown that the mechanical vascular obstruction, mast cell accumulation, inflammation and fibrosis may be ameliorated using the renin angiotensin aldosterone system modulators including losartan, captopril, and aliskiren,[1, 16, 18] presenting a potential target for therapy development.[8] In our study emboli resolved spontaneously in a short period of time. Therefore, the retinal damage may be primarily due to the inflammatory and endothelial damage than from ischemia. This may also mean that either preventing fat emboli

in the retina or targeting early stages in the pathophysiology may be more effective than trying to speed their destruction. Many treatments have been used to treat the early stages of FES, but the most consistently effective has been corticosteroids. Corticosteroids limit free fatty acid levels, stabilize membranes, and inhibit leukocyte aggregation.[13, 17]

In this study, significant trends in emboli size or position in the retinal layers were not found. Previous studies have shown that the retinal ganglion cell layer is especially susceptible to hypoxia, but in this study, we did not find that emboli preferentially localized to any layer.[19, 20] Sample size and the time interval may have influenced this outcome. Neutral fat has been proven to be the cause of FES. Therefore, the triolein model is thought to provide a reliable model for the disease. Numerous other models have been used to study hypoxia in the retina including a study by Lee that used surgical occlusion of the carotid arteries.[20, 21] Similar histopathological findings have been observed in the lungs of patients with FES and those of rats injected with triolein.[14]

However, the fat emboli released during bone trauma also contain the cellular component of the bone marrow.[2]

This research furthers the understanding of the timeline of the FES disease process. Although there are other rat models for hypoxia, this model allows a less invasive method to observe hypoxic changes in the retina and future studies could examine the signaling changes in the retina after this process.[21] Developing an understanding of how hypoxia changes cellular signaling pathways in the retina can have far reaching consequences into many diseases processes and provide possible targets for pharmacologic study.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paula Monaghan Nichols, Alan Poisner, Agostino Molteni and Peter Koulen conceived and designed the experiments; Caroline G. Olson and Landon J. Rohowetz performed the experiments; Caroline G. Olson, Landon J. Rohowetz, and Peter Koulen analyzed the data and wrote the paper. All authors edited and reviewed the paper.

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