Available online at https://li02.tci-thaijo.org/index.php/ssstj

Suan Sunandha Science and Technology Journal

Comparison of the Effectiveness of Two Histochemical Staining Techniques for Steatosis Detection in Liver Tissue and Application in Forensic Autopsy: A Case Study

Yasudama Chaimad[1*], Woratuch Witchuvanich[2]

[1]Program in Forensic Science and Criminal Justice, Faculty of Science, Silpakorn University, Mueang Nakhon Pathom District, Nakhon Pathom Province 73000, Thailand
[2]Faculty of Forensic Science, Royal Police Cadet Academy, Sampran District, Nakhon Pathom 73100, Thailand
***Corresponding author e-mail: yasudama2929@gmail.com**

Received: *1 February* 2023 / **Revised:** *21 March 2023* / **Accepted:** *19 April 2023*

Abstract

Oil Red O (ORO) stain is lipophilic for stain fat and lipid components, it is one of the chemical reagents which have the potential to be utilized for forensic medicine to identify asphyxia deaths from pulmonary emboli, that are frequently occurred following an accident or sudden death. Consequently, it can increase forensic pathologists' confidence and lower the cost of delivering ORO stains to other units. Therefore, the authors were interested in comparing the effectiveness of commercial ORO-C and homemade ORO-HM in steatosis samples. Tissue samples were collected and divided into two groups namely with fixative and without any preservative. Assessment of the persistence efficiency of ORO-HM at different storage periods was performed by four experts with a blind testing method. The results were compared by assessing the efficiency of two histochemical stains using the SPSS software. The results showed that ORO-HM in both fresh and formalin-fixed tissues represented decent efficiency in fatty changed detection which was in line with the results from ORO-C. However, the ORO-HM data in formalin fixative tissues was clearer than fresh tissue ($p < 0.05$). This study presents that the ORO-HM method expresses promising results, that is useful for the diagnosis of steatosis as same as ORC-C. Furthermore, it should be performed in slides of no longer than 72 h storage time because the efficiency of the dye would deteriorate significantly. In conclusion, ORO-HM stain in this study provided compatible results with PRC-C and could be utilized in forensic medicine.

Keywords: Fatty change, Oil Red O, Fat embolism, Histochemical staining, Forensic medicine

1. Introduction

Oil Red O (ORO) is a lysochrome (a fat-soluble dye) diazo dye uses for staining of neutral triglycerides, fatty acids, and lipids on frozen sections and some lipoproteins on paraffin sections. Alexandre Beaudoin discovered it in 2004, and its structural formula is $C_{26}H_{24}N_4O$ (Figure 1) (Bumbrah, Sodhi, & Kaur (2019). ORO stains complex phospholipids and glycolipids with polar groups poorly, which means it does not stain myelin, peripheral nerves, or biological membranes and thus highlights only fat droplets. The staining has to be performed on fresh samples, as alcohol fixation removes most lipids (Definition of Oil Red O, 2021). Some benefits of ORO stains; pathologists use ORO stains in muscle biopsies to assess the amount of sarcoplasmic lipid droplets and to look for lipid storage diseases, primary carnitine deficiency, neutral lipid storage disease with myopathy, assessing steatosis in liver transplant biopsy (Riva et al., 2018). It was used in cytopathology to raise the levels of lipid-laden macrophages (Quan, Hoerger, Mullins, & Kuhn, 2022), in lung transplant biopsies (Marangu et al., 2018), various respiratory conditions, including chronic smoking, gastroesophageal reflux (Hopkins et al., 2010), lipoid pneumonia (Nguyen & Oh, 2013), fat embolism, pulmonary alveolar proteinosis and pulmonary aspiration (Bandla, Davis, & Hopkins, 1999). In clinical pathology, it is used to fecal fat test which is a test for indicating abnormal fat absorption (Fine & Ogunji, 2000) and study lipid metabolism in worms (Wang & Ching, 2021). In addition to forensic science or forensic medicine, ORO is used for fat emboli detection in tissue (Milroy & Parai, 2019; Turkmen Samdanci et al., 2019) or the

Suan Sunandha Science and Technology Journal

development of latent fingerprints on porous exhibits that are dry or wet, such as paper, cardboard (Bumbrah et al., 2019), etc.

In current practice, forensic histopathology laboratory of Institute of Forensic Medicine Police hospital, has a role to support the work of forensic medicine in determining the cause of death. Based on the principles of histology and pathology. As mentioned above, in forensic science, fat emboli detection techniques are commonly used in cases of unexpected death or sudden death when the autopsy and the determination of the cause of death by the hematoxylin and eosin stain technique (H and E) cannot explain the exact cause of death. Fat emboli can be seen in other organs, including the brain, kidneys, and heart muscle (Turkmen Samdanci et al., 2019). There is a mechanism by fat droplets obstruction of blood vessels and / or by biochemical methods, i.e. lipolysis into free fatty acids induce an inflammatory response (Parai & Milroy, 2018). For instance, death from cardiopulmonary resuscitation (CPR) is an emergency procedure for maintaining blood circulation and oxygenation during cardiac arrest (Deliliga, Chatzinikolaou, Koutsoukis, Chrysovergis, & Voultsos, 2019), Lipoplasty or liposuction, the surgical process of removing excess fat or orthopedic surgery. Trauma in general, and particularly fractures of long bones or ribs (after cardiopulmonary resuscitation (CPR)) or accident and intra-medullary nailing (Voisard, Schweitzer, & Jackowski, 2013), Blunt injury of adipose tissue, fatty liver and pancreatic necrosis as well as other factors such as severe toxicity that causes liver failure are additional suspected or proven triggers of fat embolism. Furthermore, it can also be seen in sudden deaths caused by a fat embolism in the lungs in patients with miliary tuberculosis, which 90% of the people with incidents such as those listed above showed a microscopically visible fat embolism. (Chinen & Ito, 2019). Therefore, detection of a fat embolism in autopsy requires the history, clinical and laboratory findings along with autopsy investigations to determine its relevance.

According to data from the Institute of Forensic Medicine Police Hospital, there are approximately 5,600 autopsies performed each year. In 3,350 cases (accident 1,306 cases), an unknown cause of death was discovered. To support forensic medicine practice and reduce the cost of sending ORO to external agencies. Therefore, the researcher is interested in preparing a histochemical stain for fat emboli detection called ORO in-house (ORO-Homemade, ORO-HM), with the aim of comparing the efficacy of ORO in-house with ORO commercial (ORO-C stain sets). As far as we know, it has been recommended that tissue stained with Oil Red O must be examined within 24 hours because the quality of the ORO will fade due to lipids that may be accused of distorting and floating together because of the lower surface tension of the lysochrome organic solvent. Therefore, the researcher was interested in studying the stability of the ORO-HM dye at 48 h, 72 h, 7 days, 1 mo, and 2 months. If the lipid drops in Oil Red O stained tissue, it will keep its original position and color over time. (Christoffersen & Thomsen, 2014).

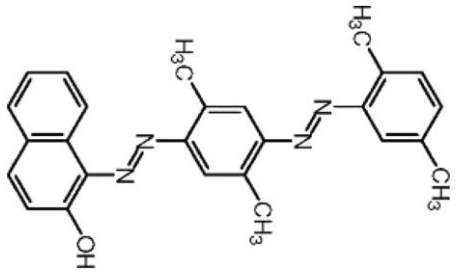

Figure 1. Schematic representation of the structure formula of Oil Red O ($C_{26}H_{24}N_4O$) (Bumbrah, Sodhi, & Kaur (2019).

As a result, the goal of this study is to (1) compare the quality of fat stains in tissue between the ORO-HM and ORO-C stain sets. (2) To assess the ability to report the diagnosis of steatosis or a fatty change in tissue between the ORO-HM and ORO-C stain sets, (3) To assess the persistence of ORO-HM at different time intervals.

2. Materials and Methods

2.1 Chemicals

All reagents used were at least analytical reagent (AR) grade. Isopropyl alcohol was supplied by Lab scan (Thailand). Oil Red O-HM was supplied by Sigma-Aldrich (Thailand) and Oil Red O–Commercial set was supplied by Bio-Optica. 40% formaldehyde was supplied by the Government Pharmaceutical Organization. O.C.T. Compound embedding medium for cryostat was supplied by Bio-Optica. Mayer Hematoxylin was supplied by Thermo Fisher Scientific (Thailand).

Suan Sunandha Science and Technology Journal

2.2 Instruments

Embedding tissue was performed by Medite model TES Valida, Microtome section was done by Epredia™ model HM 355S, Automatic slide strainer was Intelsint model AUS-1, Microscope was Nikon model Ni-U and Cryostat Microtome (Frozen section) was Leica model CM1860 UV Cryostat in which temperature range of 0°C to -60°C was used for frozen fresh tissue.

2.3 Methods for preparing reagents and ORO-HM dyes

Methods for the preparation of ORO-HM dyes, (a) Preparation of Oil Red O stock solution, 0.5g of Oil Red O powder was dissolved with 100 ml of isopropyl alcohol and mix until homogeneous achieved; (b) preparation of Oil Red O working solution, 3 ml of stock Oil Red O with 2 ml of distilled water mixed well and leaved at room temperature for about 30 min; and (c) filter before used; this dye cannot be reused.

Methods for 60% isopropyl alcohol, 30 ml of isopropyl alcohol was measured and mixed with 20 ml of distilled water.

Methods for the gelatin jelly, 10 g of gelatin weighted and mixed with 52.5 ml of distilled water, melted by heat and added glycerin and phenol when it was completely dissolved. Mix together and leave to cool to form a jelly. It was Preserved in the refrigerator.

2.4 Samples / study population

This research was a case-control study. Samples of cadavers' tissues had been granted by the Institute of Forensic Medicine, Police Hospital. All samples were blinded for ethical issues. The study was approved by the Human Research Ethics Committee Silpakorn University Certificate with issue number of COE 63.0325-024. By collecting samples from liver organs, criteria for consideration were included such as the appearance of a fatty liver or fat accumulation in the liver cells, the appearance of the surface as bulges of various sizes or smooth, yellowish-green, non-rotten condition and with rotten tissue being the exclusion criterion**.** Evaluation of pathological characteristics by a pathologist were performed by dividing into 2 types, liver tissue fixed in 10% formalin and fresh tissue samples. All tissue samples were stored at 4-8°C until analysis. The samples were collected between 14 May 2022 to 31 May 2022 with a total number of 30 cases.

- Preparation of liver tissue samples was done by cutting the liver tissue samples into 2 pieces with a sizing of 0.5x0.5x0.5 mm. Then fixed them with a desired preservative 1 piece and left the rest for a fresh sample group.
- Normal liver tissue samples or no fatty liver tissues were used as a negative control group.
- Positive fatty changes in liver tissue samples were used as a positive control group.

In assessing the quality of both sets of dyes, four experts were invited to assess the quality of both sets of dyes by random blind testing procedures on the following issues: (1) assess the quality of the fat stain; (2) ability to detect changes of fatty changes in the liver tissue of patients with steatosis; (3) evaluate the durability of ORO-HM dyes over time; and (4) suitability of ORO-HM dye to be used in a forensic medicine.

2.5 A comparison of the fat stain quality and ability to diagnose steatosis between the ORO-HM and ORO-C stain sets

2.5.1 Tissue preparation by histological technique

(a) Dissected tissue of the desired size was placed into the tissue cassettes, and then brought it into the automatic tissue processing according to the machine's working program which were as follows: (1) tissue fixation with 10% formalin, dehydration with 95% alcohol and ethanol, (2) clearing tissue sample with xylene simmering, and (3) infiltration with paraplast. Total time required was approximately 22 to 24 hours; and (b) hematoxylin and eosin staining (H and E) process was started with an automatic slide staining machine according to the machine's working program as follows:(1) deparaffinization with xylene simmering, (2) rehydration with ethanol and 95% alcohol, (3) hematoxylin nuclear staining and eosin counterstaining, (4) dehydration with 95% alcohol and ethanol. In order to make sure that the slices were clear, the final step was utilized with the process of sample clearing with xylene. Total time was roughly 30 minutes.

2.5.2 Procedure for staining liver slides with ORO

Firstly, the fresh liver and liver were fixed by 10% formalin with a sizing of 0.3x0.3x0.2 mm then they were sliced into thin tissues by a cryostat machine. The obtained slides were then stained with

Suan Sunandha Science and Technology Journal

Oil Red O according to the following procedure. (1) Oil Red O homemade (ORO-HM) staining, (2) the slides were dipped in 60% isopropyl alcohol and (3) stained with working Oil Red O for 20 minutes before being differentiated in 60% isopropyl alcohol until the background color was completely removed and (4) washing with running tap water. (5) The slide was then counterstained with hematoxylin for 1-2 minutes before being washed for 5 minutes under running tap water. In the final process, slides were wiped dry and coated with gelatin jelly glass lacquer, a solution used exclusively for tissue lipid staining.

Secondly, standard Oil Red O commercial staining sets (ORO-C stain sets) were as follow; (1) slice section was submerged into distilled water, (2) place the ORO solution in a coplin jar, and (3) immerse the slides for 20 minutes before washing quickly in tap water. (4) Drain the slide and place it on hematoxylin solution for 30 seconds before washing in running tap water for 3 minutes, Final step was to drain and mount in gelatin jelly.

2.6 Assessment of ORO-HM dyes persistence over time

Take slide sections from point 2.5 of each sample were examined and photographed at x40 magnification within 24 h after staining. After that, all slides were stored in small plastic boxes. At the same time, all of the samples were analyzed, and they were all kept at the same steady ambient temperature in a dark location. examination and photography were repeated, using the same microscope at 24 h, 48 h, 72 h, 7 days, 1 month, and 2 months, respectively. Each time, photos of the identical area of each sample were taken. Finally, the pictures were compared in order to ascertain if the lipids in each sample had migrated from their initial positions as well as to assess the color quality.

2.7 Statistical analysis

The data were expressed as the mean and comparative mean differences in fatty tissue staining ability and the reporting of hepatic steatosis between ORO-HM stain and ORO-C stain sets, and the stability color of the ORO-HM stain was assessed using the SPSS program. The analysis was performed by one-way ANOVA and Chi-square tests. Differences were considered to be statistically significant at $p < 0.05$.

3. Results and Discussion

3.1 Assessment of hepatic steatosis samples by hematoxylin and eosin (H and E) methods

Hepatocytes, which are large polygonal cells with eosinophilic (pink) cytoplasm and round nuclei, present in Figure 2a as histopathological features of the normal liver. The portal tracts (PT) consist of the hepatic artery (HA), portal vein (PV), and bile duct (BD). The CV is lined by a single layer of endothelial cells and drains blood coming from the portal tracts (PT) via sinusoids. Hepatocyte plates extend toward the portal tracts from the central vein (CV) (PT) in the picture on the right. Histopathological features of the abnormal liver demonstrated fatty changes or steatosis, which represents the intracytoplasmic accumulation of triglycerides. The hepatocytes present small fat vacuoles and macrovesicular fatty change (Figure 2b) (Liver Fellow Network, 2020).

A total of 30 liver tissue samples were examined using hematoxylin and eosin (H and E) staining by two pathologists. According to histology, 16 cases of steatosis show liver parenchyma with markedly diffuse small and large droplet infiltration, no inflammatory cell or malignancy is seen. In addition, fibrosis of the liver parenchyma and cirrhosis were also found, and 14 cases of non-steatosis show liver parenchyma composed of small lobules of roughly hexagonal shape with portal tracts at the apices. Inside the lobules, the hepatocytes are arranged as cords of cells connecting the portal tracts in the periphery to the central veins. In addition, in some cases, liver cells are also found to be inflamed.

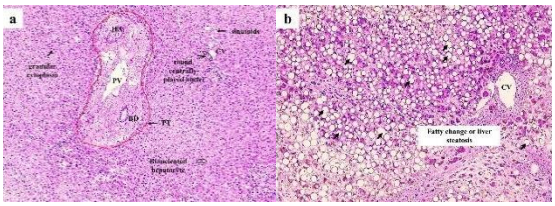

Figure 2. Histopathological comparison images of liver tissue samples. a) normal liver showed the central vein (CV), portal tracts (PT), hepatic artery (HA), portal vein (PV) and bile duct (BD) b) abnormal liver showed fatty changes or steatosis (shown with arrows, 10x).

Suan Sunandha Science and Technology Journal

3.2 Assessment of quality of fat stain between ORO-HM and ORO-C stain sets

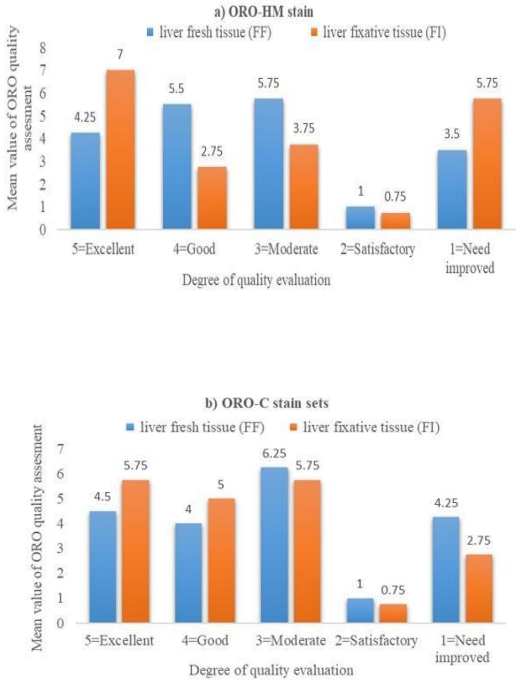

Figure 3. The mean qualitative assessment results between a) ORO-HM and b) ORO-C stain sets in the liver samples for both types, (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue).

Figure 3 shows the average results of the quality evaluation of the two ORO lipid dyes for both types. Four experts evaluated liver samples based on the type of specimen as follows: 3a) presented, the staining quality of ORO-HM in the fresh liver sample showed excellent staining quality of ORO-HM 4.25 (21%), good 5.5 (27%), moderate 5.75 (29%), satisfactory 1 (5%), and needs improvement 3.5 (18%), respectively. Likewise, ORO-C stain set lipid quality in non-fixed liver samples showed that the staining quality was excellent 4.5 (23%), good 4 (20%), moderate 6.25 (31%), satisfactory 1 (5%) and need improved 4.25 (21%). As for the fixative liver samples, the staining quality of the ORO-C stain set was excellent 5.75 (28%), good 5 (25%), moderate 5.75 (29%), satisfactory 0.75 (4%) and need improved 2.75 (14%), respectively.

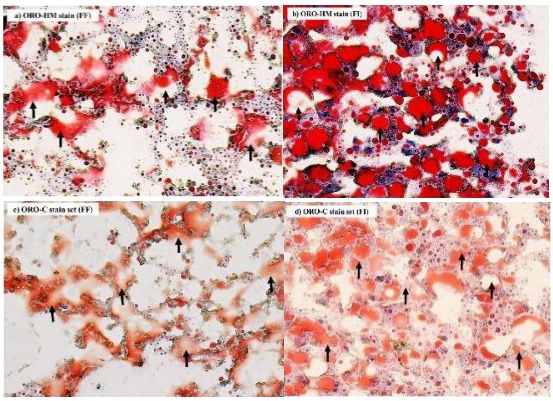

Figure 4. Photographs of liver fatty changes by ORO-HM and ORO-C stain sets between fresh and fixative tissue samples a) ORO-HM stain (fresh tissue), b) ORO-HM stain (fixative tissue), c) ORO-C stain sets (fresh tissue) and d) ORO-C stain sets (fixative tissue) x10 magnification., (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue).

Figure 4, shows the histopathology of liver steatosis by the histochemical stain in 4 groups. Morphologic and shade of color differentiation was easily assessed the fatty change using the ORO-HM stain (fixative tissue) and the ORO-C stain sets (fixative tissue). Unlike the ORO-HM (fresh tissue) stain, the ORO-C (fresh tissue) stain series is difficult to diagnose due to the altered shape of the liver cells. Poor nuclear staining obscures hepatocyte boundaries. In Figures 3 and 4, it can be seen when liver steatosis was stained with ORO-HM and ORO-C stain sets in fresh autopsy samples, the quality was not different at all levels, i.e., excellent at 4.25 and 4.5, good at 5.5 and 4.0, moderate at 5.75 and 6.25, satisfactory at 1 and 1, and need improved at 3.5 and 4.25, respectively. However, the ORO-C stain sets had a higher mean quality improvement than the ORO-HM. This was due to the non-fixative in tissue specimens, which caused specimens to be easily degenerative, difficult to frozen section, easily folding and wrinkled, and deteriorating the nucleus structure. When examined under a microscope, the nuclei were not clearly stained, and cell boundaries were unclear. As illustrated in Figures 4a) and 4c), this makes fatty change diagnosis difficult. In contrast, when staining liver steatosis with ORO-HM and ORO-C stain sets in fixative specimens, the quality of the stain was consistent with fresh tissue samples at all levels; thus, excellent was 4.25 and

Suan Sunandha Science and Technology Journal

4.5, good was 5.5 and 4.0, moderate was 5.75 and 6.25, satisfactory was 1 and 1, and need improved was 3.5 and 4.25, respectively, but the fixative tissue samples were able to diagnose fatty change more easily than fresh tissue samples, as shown in Figures 4b and 4d.

Table 1 shows the results of the statistical analysis of fat stain quality between ORO-HM and ORO-C stain sets on liver tissue slides. When comparing ORO-HM in fresh tissue samples, it was found that the quality had no difference at all levels with ORO-C stain sets in both fixative and fresh. However, when only comparing ORO-HM, found that ORO-HM in fresh tissue samples showed significantly different dye quality at every level from ORO-HM in fixative tissue samples. Statistically, the value is 0.009 ($p < 0.05$).

Table 1. The statistical analysis of qualitative assessment results between ORO-HM and ORO-C stain sets on liver tissue slides.

Type of ORO	Type of ORO	Mean Different	Std. Error	Sig.
ORO-HM (FF)	ORO-C (FF)	-.300	.227	.187
	ORO-HM (FI)	-.600*	.227	.009*
	ORO-C (FI)	-.425	.227	.062
ORO-C (FF)	ORO-HM (FF)	.300	.227	.187
	ORO-HM (FI)	-.300	.227	.187
	ORO-C (FI)	-.125	.227	.582
ORO-HM (FI)	ORO-HM (FF)	.600*	.227	.009*
	ORO-C (FF)	.300	.227	.187
	ORO-C (FI)	.175	.227	.441
ORO-C (FI)	ORO-HM (FF)	.425	.227	.062
	ORO-C (FF)	.125	.227	.582
	ORO-HM (FI)	-.175	.227	.441

*The mean difference is significant at the 0.05 level., (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue)

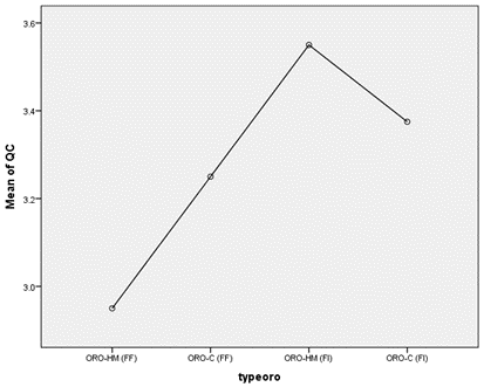

Figure 5. The means plot of qualitative assessment results between ORO-HM and ORO-C stain sets in the liver samples for both types., (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue).

Figure 5 shows the average results of four experts who evaluated the quality of the two ORO lipid dyes for both types of liver samples as follows: they concluded that the best quality staining of ORO is ORO-HM in fixative tissue samples (ORO-HM (FI)) = 3.55, ORO-C stain sets in fixative tissue samples (ORO-C (FI)) = 3.38, ORO-C stain sets in fresh tissue samples (ORO-HM (FF)) = 3.25 and ORO-HM in fresh tissue samples (ORO-HM (FF)) = 2.95, respectively.

Although the qualitative assessment results of the four lipid dye groups in parts of their frequency distribution (Figure 3) and histopathological results are shown in Figure 4, did not differ at all levels of qualitative assessment, it is clear that all four levels of quality are trending in the same direction. ORO-HM results for fresh and fixative tissue samples are excellent (4.25 and 7.0), good (5.5 and 2.75), moderate (5.75 and 3.75), satisfactory (1.75 and 1.0), and need to be improved at 3.5 and 5.75, respectively. ORO-C stain sets, both fresh and fixative tissue samples, yielded excellent results of 4.5 and 5.75, good results of 4.0 and 5.0, moderate results of 6.25 and 5.75, satisfactory results of 1.0 and 0.75, and needed improvements of 4.25 and 2.75, respectively. From the study results, it can be seen that both the ORO-HM and ORO-C stain sets had a relatively high level of need for improvement, and changes in the structure of the nucleus, and cell boundaries are unclear, which interferes with the detection of fatty changes. However, when evaluating the quality of the four dye groups using one-way ANOVA statistics, the results were found to be in the same direction as the frequency distribution for only the three groups, which is the quality of ORO-HM dyes for both fresh and fixative tissue samples. There was no difference at all levels when compared with ORO-C stain sets. However, ORO-HM in fixative tissue samples showed different levels of quality than ORO-HM in fresh tissue samples, as shown in Table 1, in which ORO-HM fixative tissue samples showed better staining quality than ORO-HM in fresh tissue samples, and ORO-C stain sets in both fresh and fixative tissue samples are shown in Figure 5. Therefore, in order to make a reliable diagnosis of steatosis with ORO dye, ORO-HM can be used in fixative specimens because the staining quality is similar to that of ORO-C stain sets.

3.3 Assessment of the diagnostic competency of steatosis between ORO-HM and ORO-C stain sets.

According to the results of the detection of fatty changes on liver steatosis slides stained with ORO-HM, the positive results ranged from mild to severe, with the most moderate yield being 56.9%, while the positive effects of the ORO-C stain sets were also

Suan Sunandha Science and Technology Journal

positive in the mild to severe range, with the most severe effect being 54.7%. As shown in Table 2.

Table 2. Comparison of levels of diagnostic competency for fatty liver disease between ORO-HM and ORO-C stain sets.

Tissue slides	ORO type		Grading steatosis				
			Not found	Mild	Moderate	Severe	Total
Liver steatosis	ORO-HM	Count	66	22	33	31	160
		%Grading steatosis	53.2 %	42.3 %	56.9 %	45.3 %	50 %
	ORO-C stain sets	Count	58	30	25	47	160
		%Grading steatosis	46.8 %	57.7 %	43.1 %	54.7 %	50 %

(ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue)

Table 3. Statistical analysis of fatty changes detected on liver tissue slides.

	Value	df	Asymp. Sig. (2 sides)
Pearson Chi square	3.595[a]	3	.309
Likelihood Ratio	3.604	3	.307
Linear-by-Linear Association	.662	1	.416
N of Valid Cases	320		

Chi-squared test, *p*-value < 0.05 statistically significant., (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (ORO-C stain sets, ORO-C), (FF, fresh tissue or non-fixative), (FI, fixative tissue)

The results of statistical differential analysis by SPSS using the chi-square test showed that the fatty changes of ORO-HM and ORO-C stain sets in liver steatosis tissues were not significantly different at the 0.05 level, as shown in Table 3.

3.4 Evaluation of ORO-HM dye quality stability over time

The image shows the persistent quality of ORO-HM dye in fresh and fixative tissue samples over time. It was discovered that the dye's quality can be found to be good for a period of 24 to 72 hours. Macrovesicular steatosis has a single, large vacuole of fat with clear boundaries, which are indicated with black arrows. The color persistence quality of ORO-HM in fresh and fixed tissue samples from day 7 to 2 months showed a deterioration in quality, small fat droplets were found scattered throughout, and patchy artifacts were indicated with yellow arrows. Specifically, in the fresh tissue samples, agglutination of fat droplets (red circles) was observed. The hepatocyte boundaries were not clear; the color was faded, but in the fixative tissue specimen, the appearance of the fat vesicle was still evident. The cell boundaries were still evident, and the color has faded slightly.

The image presented in Figure 6 is an example taken from the same sample that was stored in the same slide box with no moisture and not exposed to sunlight, it was used to check the persistence quality of ORO-HM dye by collecting data for 24 h, 48 h, 72 h, 7 days, 1 month, and 2 months, respectively. Based on previous knowledge, it is recommended that fatty changes should be read immediately or no later than 24 hours for the diagnosis of fatty changes with ORO, which provides the most reliable diagnosis (Christoffersen & Thomsen, 2014).

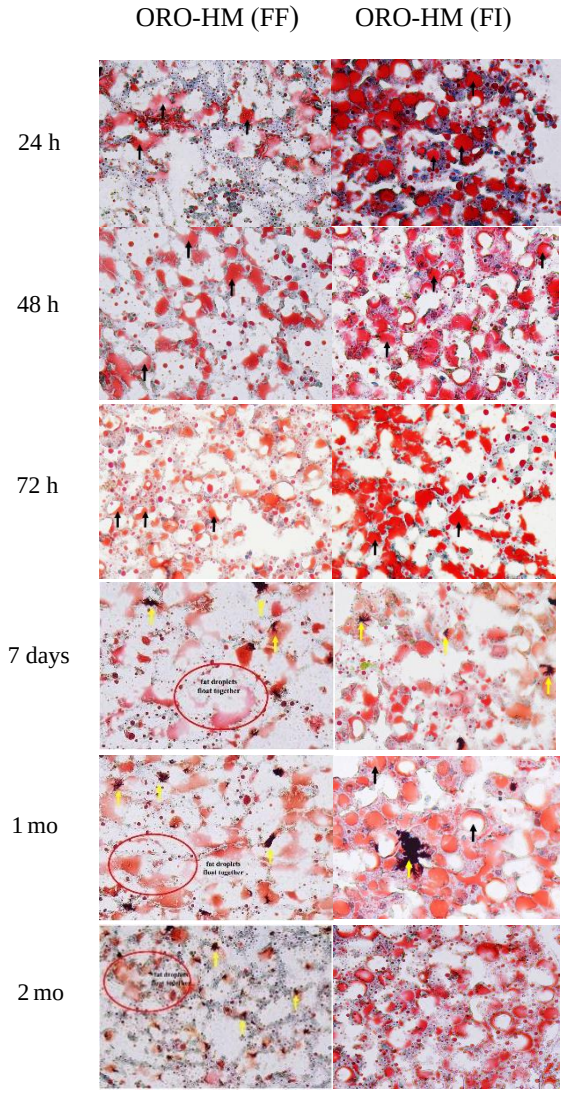

Figure 6. A section stained with Oil Red O-HM in the liver samples for both types followed over time., (ORO, Oil Red O), (ORO-Homemade, ORO-HM), (FF, fresh tissue or non-fixative), (FI, fixative tissue).

From the experiments, it can be seen that ORO-HM can be used to detect fatty changes well in 24 to 72 hours, and the slides should not be reinterpreted when stored for more than 1week because the color quality decreases and there is a precipitation. From

the experiments, it can see that ORO-HM can use to detect fatty changes well in a period of 24 to 72 hours, and the slides should not be reinterpreted when stored for more than 1 week because the color quality decreases and there is a precipitation. There is an agglomeration of pigments. The color of the nucleus faded, and there were scattered fat droplets, which hampered interpretation of the results. To determine the effectiveness of the fat stain, all ORO-HMs were found to be consistent with the quality and reportability of the results, i.e., the ORO-HM stain could be used for the diagnosis of steatosis in fixative tissue samples, which provides good quality and can extend the color sticking life for a long time.

3.5 Apply ORO-HM stain in forensic autopsy: Case study

Female, 29 years old, overweight, cause of autopsy due to unnatural death from undergoing breast augmentation liposuction surgery at a clinic. Dead at the hospital, have a history of resuscitation. The first hospital performed an autopsy and found that the cause of death was due to sepsis or septicemia. However, relatives are still suspicious about the cause of death. Therefore, they offered to have the police freeze the corpses and sent the corpses to the second hospital for a thorough autopsy again ("Relatives are fascinated by a woman", 2020)

Autopsy finding, the condition of the body was examined. The external examination of the corpse found wounds on the upper right arm, left arm, under the left breast, right leg, right leg, and left leg. There were no abnormalities discovered during an internal corpse examination of the head, neck, and abdomen. A large bruise wound in the chest spread across the chest area. Tissue samples were sent to the laboratory for hematoxylin and eosin and Oil Red O stain techniques.

Histopathology findings by hematoxylin and eosin methods found that the brain has subcerebral hemorrhage, edema, neuron degeneration, and cell death. Congestive heart edema and inflammation, lungs hemorrhage in the alveoli. The liver and pancreas found fat infiltrates in cells and acute kidney failure. The uterus and ovary had some hemorrhagic patches. ORO-HM stained in lung autopsy showed fat emboli positive within the alveolar space and small patches along the alveolar wall (Figures 7 c and 7d). In addition, they found blood clots within the alveoli of the lungs.

Although death from fat emboli can be tested in multiple organs such as the brain, heart, liver, or kidney in forensic medicine, the lung is the most appropriate organ to use because it is an organ related to the respiratory system and can explain the death most clearly (Turkmen Samdanci et al., 2019). Figure 7 shows the histopathological results of the case study in that the pathologist who sent the stain for histological examination used the Oil Red O color. The laboratory uses a lung fixative tissue autopsy sample for testing. Figures 7c and 7d demonstrate that the ORO-HM dye clearly stained both small and large fat droplets within the alveoli, and found blood congestion in the alveoli that was spread throughout the lung tissue. It is assumed that the mechanism of death in the case study was caused by fat droplets from the liposuction process that escaped into the circulatory system and eventually obstructed the trachea and caused asphyxia. The liver tissue was used as a negative control and a positive control, as shown in Figures 7a and 7b, respectively.

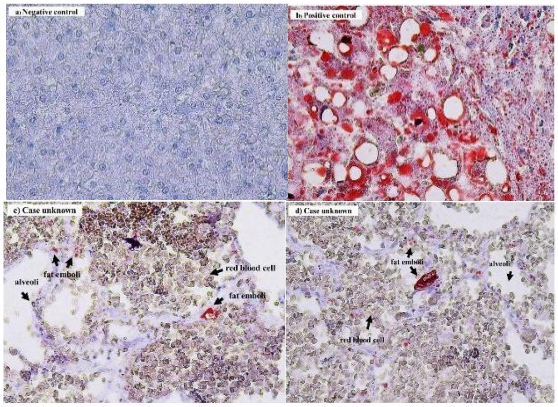

Figure 7. Frozen section autopsy microscopy in samples a) liver: negative control ORO-HM (10X), b) liver: positive control ORO-HM and c and d) lung: Oil red O staining showing large and small fat droplets within the alveolar space (40X)., (ORO, Oil Red O), (ORO-Homemade, ORO-HM).

4. Conclusions

A comparative study of dye efficacy between ORO-homemade (ORO-HM) and commercial ORO (ORO-C stain sets) was performed using liver tissue samples with steatosis and normal liver pathology. A comparison was also made between fresh and fixative liver tissue samples, which was characterized by the samples which were confirmed by a pathologist. The study consisted of assessing the quality of ORO to detect hepatocyte fatty changes, which rating on a 4 scale: excellent, good, moderate, and needing improvement. It was found that the staining quality of fatty changes in hepatocytes in both fresh and fixative tissue samples with ORO-HM was not different from that of ORO-C stain sets. However, ORO-HM in fixative tissue samples had better staining quality than ORO-HM in fresh liver samples. Because fresh tissue samples have a chance to rot easily, the cellular structure changes to an unclear extent. The nucleus begins to partially die. When considering the persistence and quality of ORO-HM in liver cells in both fresh and

fixative tissue samples at different times were examined, it was discovered that ORO-HM stained liver cells from 24 to 72 hours can best fatty changes in the liver in the better condition. Compared with the time period of 1 week to 2 months, the persistence of the ORO-HM dye was observed in both fresh and fixative tissue samples and markedly decreased. Especially in ORO-HM fresh tissue samples, fatty changes fusion was found, cell nuclei deteriorated, and cell boundaries were not clearly visible. In addition, in both ORO-HM in fresh and fixative tissue samples, the quality of the dye is faded color and contaminated, which interferes with the diagnosis. The final efficacy assessment of the ORO stain was its ability to report steatosis, found that the ORO-HM stain detects fatty changes at levels of severe steatosis (45.3%) less than ORO stain sets (54.7%) and can report cases where fatty changes were not found at 53.2% and 46.8%, respectively. When the correlation was analyzed by SPSS, it was found that ORO-HM's ability to diagnose steatosis was not different from that of ORO-C stain sets. Therefore, when the ORO-HM dye was used to explain the cause of death in a case study where the preliminary autopsy results could not find a cause of death, Analysis of the cause of death with histochemical staining techniques can detect fat droplets inserted within the alveoli and spreading to different parts of the lung tissue. This technique can support the doctors concluding the cause of death in the case study was due to circulatory failure and asphyxiation from fat droplets becoming obstructed in the trachea, a condition known as a fat embolism. Thus, from all studies, it can be concluded that a fat dye prepared in-house in the laboratory (ORO-HM) is as effective as ORO-C stain sets for elucidating the cause of mortality in unexplained cases. However, the tissue samples should be fixative, and the result should be diagnosed within 3 days to make the interpretation accurate and the most reliable.

Acknowledgements

We would like to express our gratitude to all three experts as well as Police Colonel Pakorn Wisinrat, Deputy Commander of the Institute of Forensic Medicine. Police Hospital, Police Lieutenant Ratchakorn Anantapada, Doctor Pathologist at Pathology Division, Police Hospital, and Dr. Tharinthorn Chansoon, Head of Histopathology Laboratory, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, and we would like to thank the forensic histopathology laboratory at the Institute of Forensic Medicine, Police Hospital, and the pathology laboratory in the Police Hospital for their support and excellent work next to their daily routine and some other authors cited in the text for making literature accessible and for the stimulating discussions.

Ethical Approval

The study was approved by the Human Research Ethics Committee. From the Human Research Ethics Committee Silpakorn University (Date 25 March 2020). Number of ethics COE 63.0325-024

References

Bandla, H. P. R., Davis, S. H., & Hopkins, N. E. (1999). Lipoid pneumonia: A silent complication of mineral oil aspiration. *Pediatrics*, *103*(2), 1-4. doi:10.1542/peds.103.2.e19

Bumbrah, G. S., Sodhi, G. S., & Kaur, J. (2019). Oil Red O (ORO) reagent for detection of latent fingermarks: A review. *Egyptian Journal of Forensic Science*, *9*(3), 1-7. doi:10.1186/s41935-018-0107-1

Chinen, K., & Ito, K. (2019). Sudden death caused by pulmonary fat embolism in a patient with miliary tuberculosis. *Autopsy and Case Reports*, *9*(1), 1-10. doi:10.4322/acr.2018.059

Christoffersen, S. D., & Thomsen, J. L. (2014). Can examination of tissue stained with Oil red O be postponed up to three months? *Scandinavian Journal of Forensic Science*, *20*(2), 50-52. doi:10.2478/sjfs-2014-0005

Definition of Oil Red O. (2021). Retrieved from https://www.pathologyoutlines.com/topic/stainsoilredo.html

Deliliga, A., Chatzinikolaou, F., Koutsoukis, D., Chrysovergis, I., & Voultsos, P. (2019). Cardiopulmonary resuscitation (CPR) complications encountered in forensic autopsy cases. *BMC Emergency Medicine*, *19*(1), 1-7. doi:10.1186/s12873-019-02345-5

Fine, K. D., & Ogunji, F. (2000). A new method of quantitative fecal fat microscopy and its correlation with chemically measured fecal fat output. *American Journal of Clinical Pathology*, *113*(4), 528-534. doi:10.1309/0t2w-nn7f-7t8q-5n8c

Hopkins, P. M., Kermeen, F., Duhig, E., Fletcher, L., Gradwell, J., Whitfield, L., … McNeil, K. (2010). Oil red O stain of alveolar macrophages is an effective screening test for gastroesophageal reflux disease in lung transplant recipients. *The Journal of Heart and Lung Transplantation*, *29*(8), 859-864. doi:10.1016/j.healun.2010.03.015

Suan Sunandha Science and Technology Journal

Liver Fellow Network. (2020). *Normal liver histology 101*. Retrieved from https://liverfellow.org/post/normal-liver-histology-101

Marangu, D., Pillay, K., Banderker, E., Gray, D., Vanker, A., & Zampoli, M. (2018). Exogenous lipoid pneumonia: an important cause of interstitial lung disease in infants. *Respirology Case Reports*, *6*(7), 1-7. doi:10.1002/rcr2.356

Milroy, C. M., & Parai, J. L. (2019). Fat embolism, fat embolism syndrome and the autopsy. *Academic Forensic Pathology*, *9*(3-4), 136-154. doi:10.1177/1925362119896351

Nguyen, C. D., & Oh, S. S. (2013). A case of exogenous lipoid pneumonia. *Respiratory Care*, *58*(3), 23-27. doi:10.4187/respcare.01727

Parai, J. L., & Milroy, C. M. (2018). The utility and scope of forensic histopathology. *Academic Forensic Pathology*, *8*(3), 426-451. doi:10.1177/1925362118797602

Quan, M. A., Hoerger, J. L., Mullins, E. H., & Kuhn, B. T. (2022). A 66-year-old man with subacute cough and worsening dyspnea previously diagnosed with COVID-19 pneumonia. *Journal of Investigative Medicine High Impact Case Reports*, *10*, 1-4. doi:10.1177/23247096211055334

Relatives are fascinated by a woman who died as a result of breast augmentation liposuction, whether done by the doctor or the clinic staff. (2020). *Thairath On Line*. Retrieved from https://www.thairath.co.th/news/crime/1921503

Riva, G., Villanova, M., Cima, L., Ghimenton, C., Bronzoni, C., Colombari, R., … Eccher, A. (2018). Oil Red O is a useful tool to assess donor liver steatosis on frozen sections during transplantation. *Transplantation Proceedings*, *50*(10), 3539-3543. doi:10.1016/j.transproceed.2018.06.013

Turkmen Samdanci, E., Reha Celik, M., Pehlivan, S., Celbis, O., Turkkan, D., Ozdemir Kara, D., & Pamukcu, E. (2019). Histopathological evaluation of autopsy cases with isolated pulmonary fat embolism (IPFE): Is cardiopulmonary resuscitation a main cause of death in IPFE? *Open Access Emergency Medicine*, *11*, 121-127. doi:10.2147/OAEM.S194340

Voisard, M. X., Schweitzer, W., & Jackowski, C. (2013). Pulmonary fat embolism-A prospective study within the forensic autopsy collective of the Republic of Iceland. *Journal of Forensic Science*, *58*, 105-111. doi:10.1111/1556-4029.12003

Wang, F. Y., & Ching, T. T. (2021). Oil Red O staining for lipid content in *Caenorhabditis elegans*. *Bio-protocol*, *11*(16), 1-9. doi:10.21769/BioProtoc.4124