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Efficacy and application study of lipid stain by chromic acid techniques for determining the cause of death from a fat embolism

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ABSTRACT

Fat embolism is a common cause of death after trauma or non-traumatic cases as fat droplets may block blood vessels. It can be identified during autopsy through the microscopic examination of the lungs using Oil Red O (ORO) conducted on frozen tissue, including formalin-fixed tissue. Fat can also be identified using the chromic acid technique. Consequently, in order to increase forensic pathologists' confidence and reduce the cost of delivering ORO stains to other units, the researcher aimed to compare the effectiveness of commercial ORO (ORO-C) and homemade ORO (ORO-HM) in the chromic acid technique for steatosis tissue. We separated the samples into two groups: one stained with ORO-C stain sets and the other with ORO-HM stains. Three experts evaluated the effectiveness of chromic acid stained tissue for both types of ORO using a blind test method. The results were then compared using the SPSS program. The results showed that chromic-ORO-HM of liver had quality and efficiency in interpreting the steatosis detection, similar to the chromic-ORO-C stain sets. Histopathology findings found that fat was paler than chromic-ORO-C stain sets but showed better staining on the background. In addition, chromic acid was able to preserve fat tissue and identify fat-positive results in three victims, who died from fat emboli, suggesting it potential application in forensic medicine.

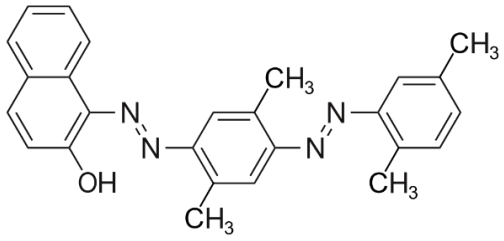
Keywords: lipid fixation; Oil Red O; fat emboli; chromic acid; forensic medicine

1. INTRODUCTION

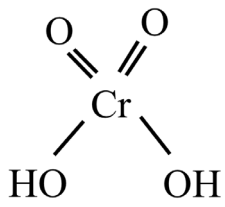
Oil Red O (ORO) is a lysochrome (a fat-soluble dye) diazo dye used to stain some lipoproteins on paraffin sections as well as neutral triglycerides, fatty acids, and lipids on frozen sections. It was discovered in 2004 by Alexandre Beaudoin ("Oil Red O," 2022), and its structural formula is $C_{26}H_{24}N_4O$. (Figure 1A). ORO does not stain myelin, peripheral nerves, biological membranes, or complex phospholipids or glycolipids containing polar groups. Pathologists use ORO stains in anatomical pathology application, such as in muscle biopsies to assess the amount of sarcoplasmic lipid droplets, primary carnitine deficiency, assessing steatosis in liver transplant biopsies, etc. (Riva et al., 2018). It was utilized in cytopathology to increase the number of lipid-laden macrophages in lung transplant biopsy samples (Quan et al., 2022; "Lipid-laden alveolar macrophage," 2020). In clinical pathology, it is used for the fecal fat test to indicate abnormal fat absorption (Fine and Ogunji, 2000) and for study lipid metabolism in worms (Wang and Ching, 2021). In addition, in forensic science or forensic medicine, ORO is used for detecting of fat emboli in tissue (Milroy and Parai, 2019; Samdanci et al., 2019) as well as for developing latent fingerprints on porous materials, whether dry or wet, such as paper, cardboard (Bumbrah et al., 2019).

In the current practice, the forensic histopathology laboratory at the Institute of Forensic Medicine, Police

hospital, has been instrumental in supporting investigations into the cause of death. This support is grounded in histological and pathological concepts. As mentioned earlier, fat emboli detection techniques are commonly used in forensic science, especially in cases of unexpected or sudden death, where the hematoxylin and eosin stain technique (H and E) during autopsy fails to elucidate the actual cause of death. Fat emboli can be seen in other organs, including the brain, kidneys, and heart muscle (Samdanci et al., 2019). There is a mechanism where fat droplets can obstruct blood arteries, and an inflammatory response may be triggered by metabolic processes, such as lipolysis into free fatty acids (Parai and Milroy, 2018). Examples include death from a bone fracture due to cardiopulmonary resuscitation (CPR) (Deliliga et al., 2019), lipoplasty or liposuction, surgical process of removing excess fat, and orthopedic surgery. In addition, trauma, and particularly bone fractures or accidents involving intra-medullary nailing, can also contribute to the occurrence of fat embolism (Voisard et al., 2013), Blunt injury of adipose tissue, fatty liver, pancreatic necrosis. 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, where 90% of the individuals experiencing incidents like those mentioned above showed a microscopically visible fat embolism (Arregui et al., 2020). Therefore, detecting a fat embolism in autopsy requires consideration of patient's history, clinical and laboratory findings along with autopsy investigations to determine its relevance.



(A) Oil Red O



(B) Chromic acid

Figure 1. Schematic representation of the structure formula of (A) Oil Red O ($C_{26}H_{24}N_4O$), and (B) chromic acid (H_2CrO_4)

In general, the most common method used to diagnose the cause of death from fat emboli is by detecting fat droplets in various organs, particularly in the lungs, using histochemical staining techniques such as ORO, Sudan Black, and Sudan III. Milroy and Parai (2019) reported that up to 93% of individuals use ORO. Based on this information, ORO staining must be performed on fresh or formalin-fixed tissue in frozen sections because alcohol fixation can remove most lipids from the tissue (Cai, 2021). Tracy and Walia (2004) have reported a method to preserve fat tissue using the post-fixative with chromic acid technique. The unsaturated fatty acids are partitioned into the tissue lipids for subsequent fixation by chromic acid, allowing paraffin-section processing for fat staining (Milroy and Parai, 2019; Arregui et al., 2020). Its structural formula is H_2CrO_4, as shown in Figure 1B ("Chromic acid," 2023).

According to data from the Institute of Forensic Medicine, Police Hospital, approximately 5,600 autopsies are performed each year. Among these, 3,350 cases (including 1,306 accidents) reveal an unknown cause of death. To support forensic medicine practices and reduce the cost of outsourcing ORO stains, the researcher is interested in developing an in-house histochemical stain for fat emboli detection called ORO homemade. This stain involves post-fixing tissue samples using the chromic acid technique (chromic-ORO-HM). Therefore, the aims of this study were to compare the quality of the fat stain in chromic acid-fixed tissue between the chromic-ORO-HM stain and chromic-ORO commercial (chromic-ORO-C) stain sets, and to assess the diagnosis efficacy of steatosis or a fatty change using chromic-ORO-HM and chromic-ORO-C stain sets, and the ability of the chromic acid technique to preserve fat tissue in three case studies.

2. MATERIALS AND METHODS

2.1 Chemicals

All reagents used were at least analytical reagent grade. Isopropyl alcohol was obtained from Lab Scan (Thailand). ORO-HM and chromic trioxide was procured from Sigma-Aldrich (Thailand) and ORO-commercial set and O.C.T. compound embedding medium for cryostat was acquired from Bio-optica (Thailand). Formaldehyde was supplied by Government Pharmaceutical Organization (Thailand). Mayer Hematoxylin was supplied by Thermo Fisher Scientific (Thailand). Linoleic acid was purchased from ICN Biochemicals, USA. Lecithin was obtained from Natural's life. Ethylene glycol and sodium bicarbonate were supplied by Mallinckrodt Pharmaceuticals, USA.

2.2 Methods for preparing reagents and ORO-HM stain

2.2.1 Preparation of ORO-HM stain

Stock ORO was prepared by dissolving 0.5g of ORO powder in 100 mL of isopropyl alcohol until homogeneous solution was obtained. Working ORO was then prepared by mixing 3 mL of stock ORO with 2 mL of distilled water, and was left at room temperature for about 30 min. The mixture solution was filtered before use. This dye cannot be reused.

2.2.2 Preparation of gelatin jelly

Ten grams of gelatin was dissolved in 52.5 mL of distilled water with heat. Glycerin and phenol were then added when gelatin was completely dissolved. The mixture was left at room temperature to cool down and allow a jelly to form. The jelly was preserved in a refrigerator.

2.3 Instruments

Tissue embedding was performed with TES Valida® paraffin embedding system, Medite model. Microtome section was carried out with Epredia™ HM 355S Automatic Microtome. Slide staining was performed using Intelsint AUS-1 automated slide stainer. Tissue observation was done with Nikon ECLIPSE Ni-U Series, upright microscopes. Tissue section was conducted using Leica CM1860 UV cryostat microtome in the temperature range of 0 °C to −60°C.

2.4 Samples/study population

This research conducted a case-control study using samples of cadaver tissues obtained with authorization from the Institute of Forensic Medicine, Police Hospital. This study was approved by the Human Research Ethics Committee (Approval number: COE 63.0325-024) at Silpakorn University. Liver tissues were collected without verified historical information. The inclusion criteria for liver tissues involved the presence of fatty liver or fat accumulation in the liver cells, with consideration of the appearance of the liver surface, such as bulges of various sizes or a smooth, yellowish-green, non-rotten condition Exclusion criteria was rotten tissue. The pathological characteristics were evaluated by a pathologist. After liver tissue was fixed by the chromic acid technique, it was divided into two types for ORO-C stain sets and ORO-HM stains. The collection period for the samples was between 14 May 2022 to 31 May 2022, comprising a total of 30 cases.

2.4.1 Sample Preparation

Liver tissue samples were cut into 2 pieces, each with a size of 0.3 x 0.3 x 0.2 mm. The samples were fixed with lipid preservative using chromic acid reagent. Normal liver tissue samples (no fatty liver) was used as the negative control group. Liver tissue samples with positive fatty changes were used as the positive control group.

2.4.2 Quality assessment and evaluation criteria

To assess the quality of both sets of dyes, three experts were participated in a random blind test to assess various aspects including: assessment of fat stain quality, assessment of the ability of both dye sets to detect fatty changes in the liver tissue of cadavers with steatosis, evaluation of the histopathology of liver tissue using hematoxylin and eosin (H&E) stains and ORO staining in tissue samples using the chromic acid technique, and application of chromic-ORO-HM dye in a forensic case study.

2.5 Comparison of the fat stain quality steatosis diagnosis

2.5.1 Tissue preparation by histological technique

Dissected tissue of the desired size was placed in tissue cassettes, and it was processed with the lipid fixative by formalin fixation, in a mixture of 70% ethylene glycol, linoleic acid, and lecithin, for 3 days at 56 °C. The tissue was rinsed several times with 70% ethanol, then several times of water for at least 8 h, before immersing the tissue in 2% chromic acid for 24 h at 4 °C followed by 24-h water rinse. Then, it was place in 5% sodium bicarbonate for 24 h, followed by water rinse for at least 8 h. The tissue was then subjected to automatic tissue processing for 22 h. The tissue section was made by paraffin blocking before staining with ORO-HM and ORO-C stain sets. Finally, permafrost slides were mounted in an aqueous medium (Tracy and Walai, 2004).

H&E staining was performed with an automatic slide stainer machine according to the machine's working program.

2.5.2 Procedure for staining liver slides with ORO

The liver tissue fixative, prepared using the chromic acid technique, was sized at 0.3 x 0.3 x 0.2 mm and sliced into thin sections by through paraffin embedding. The obtained slides were then stained with ORO according to the following procedure.

ORO-HM staining: The slides were dipped in 60% isopropyl alcohol and stained with working ORO for 20 min. Subsequently, differentiation was carried out in 60% isopropyl alcohol until the background color was completely removed. The slides were then washed with running tap water, counterstained with hematoxylin for 1–2 min, and washed for 5 min under running tap water. Finally, the slides were dried and coated with gelatin jelly glass lacquer, a solution exclusively used for tissue lipid staining.

ORO-C staining: The ORO solution was placed in a coplin jar, and the section slide was immersed in the solution for 20 min before a quick wash with tap water. Next, the slide was stained with hematoxylin solution for 30 s followed by a 3-min wash with running tap water. Finally, the slide was drained and mounted in gelatin jelly.

2.6 Application of chromic-ORO-HM dye in three forensic case studies

In this study, the researcher used the bodies of three victims, whose autopsies were requested by detectives due to their unusual deaths. The cause of death could not be determined based on the initial autopsy or by using standard examination methods. Lung tissue underwent a lipid fixative treatment using the chromic acid method and was stained with the ORO-HM and ORO-C stain sets to compare their abilities to detect fat embolism, which was suspected to be the cause of death.

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 the chromic-ORO-HM stain and chromic-ORO-C stain sets, analyzed using the SPSS program. The analysis was performed by one-way analysis of variant (one-way ANOVA), and the correlation between groups was assessed by Chi-square tests. Statistically significant differences were considered at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Evaluation of hepatic steatosis samples by H&E staining technique

Hepatocytes, characterized by large polygonal cells with eosinophilic (pink) cytoplasm and round nuclei, are shown 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 central vein (CV),

lined by a single layer of endothelial cells, and receives blood from the portal tracts (PT) through sinusoids. Hepatocyte plates extend toward the portal tracts from the CV in the image on the right. Histopathological features of the abnormal liver demonstrated fatty changes or steatosis, indicating the intracytoplasmic accumulation of triglycerides. The hepatocytes presented small fat vacuoles and macrovesicular fatty change (Figure 2B) (Bell, 2020).

A total of 30 liver tissue samples were examined using H&E staining by two pathologists. According to histology, 16 cases of steatosis showed liver parenchyma with markedly diffuse small and large droplet infiltration, with no inflammatory cell or malignancy observed. In addition, fibrosis of the liver parenchyma and cirrhosis were also found. The remaining 14 cases of non-steatosis showed liver parenchyma composed of small lobules of roughly hexagonal shape with portal tracts at the apices. Inside the lobules, 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 were inflamed.

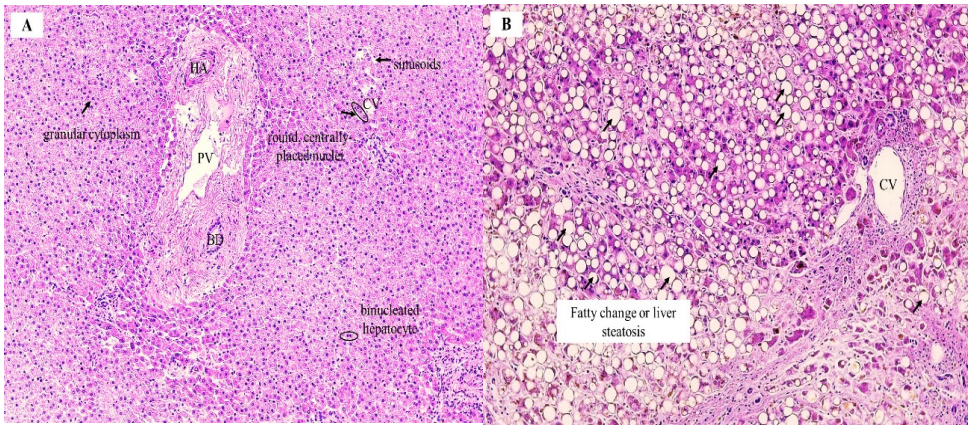


Figure 2. Histopathological comparison pictures of liver tissue samples; (A) normal liver showing the central vein (CV), portal tracts (PT), hepatic artery (HA), portal vein (PV) and bile duct (BD), and (B) abnormal liver showing fatty changes or steatosis (indicated by arrows, 10x)

3.2 Assessment of the quality of fat stain between ORO-C stain sets and ORO-HM stain by the chromic acid technique

The average results of the quality evaluation of the two ORO lipid dyes using the chromic acid technique are presented in Table 1. Three experts assessed liver samples with the same chromic acid technique. It was discovered that the chromic-ORO-C stain sets exhibited a higher mean quality than chromic-ORO-HM stain at 25% and 8%, respectively. However, on the good scale, chromic-ORO-HM stain showed a better average quality than chromic-ORO-C stain sets, with values of 20% and 40%, respectively. At other levels, the average quality of the dye was similar.

Table 1. Mean±standard deviation of fat stain qualitative assessment results between ORO-HM stain and ORO-C stain sets on liver tissue slides

Chromic-ORO type	Degree of quality					Total (n)
	Excellent	Good	Moderate	Satisfactory	Need improvement	
chromic-ORO-C stain sets	7.5±3.53 (25%)	6.0±4.24 (20%)	13.0±4.24 (43%)	3.5±3.53 (12%)	0±0 (0%)	30 (100%)
chromic-ORO-HM stain	2.5±3.53 (8%)	12.0±1.41 (40%)	12.5±0.70 (42%)	3.0±2.82 (10%)	0±0 (0%)	30 (100%)

Table 2. The statistical comparison of the quality of the chromic-ORO-C stain sets and chromic-ORO-HM stain on slides of liver tissue

Quality level		Mean different	Std. error	Sig.
Satisfactory	Moderate	.010	.157	.950
	Good	-.149	.163	.364
	Excellent	.250	.179	.166
Moderate	Satisfactory	-.010	.157	.950
	Good	-.158	.106	.138
	Excellent	.240	.129	.066
Good	Satisfactory	.149	.163	.364
	Moderate	.158	.106	.138
	Excellent	.399	.136	.004*
Excellent	Satisfactory	-.250	.179	.166
	Moderate	-.240	.129	.066
	Good	-.399	.136	.004*

Note: *The mean difference is statistically significant at the 0.05 level.

When comparing each level of quality, chromic-ORO-C stain sets and chromic-ORO-HM stain were not significantly different in moderate and satisfactory levels. However, in the good and excellent quality level, there was a significant difference in dye quality, with a statistically significant value of 0.004 (p<0.05). This implies that chromic-ORO-HM stain has a slightly lower excellent but still good quality, compared to chromic-ORO-C stain sets, as shown in Table 2.

The results of the statistical analysis by SPSS using the one-way ANOVA test showed that the quality of fat stain at all levels between chromic-ORO-C stain sets and chromic-ORO-HM stain in the liver were not significantly different, as shown in Table 3.

The findings from the average evaluation of the three experts regarding the two ORO lipid dyes for liver samples using the chromic acid technique are shown in Figure 3. The experts concluded that there was no significant difference in the quality of the two types of staining across all quality levels, with chromic-ORO-C stain sets having a mean of 3.62 and chromic-ORO-HM stain having a mean of 3.47.

Table 3. The statistical analysis of qualitative assessment results between chromic-ORO-C stain sets and chromic-ORO-HM stain on liver tissue slides

Quality assessment	Sum of squares	df	Mean square	F	Sig.
Between Groups	.675	1	.675	.855	.357
Within Groups	93.117	118	.789		
Total	93.792	119			

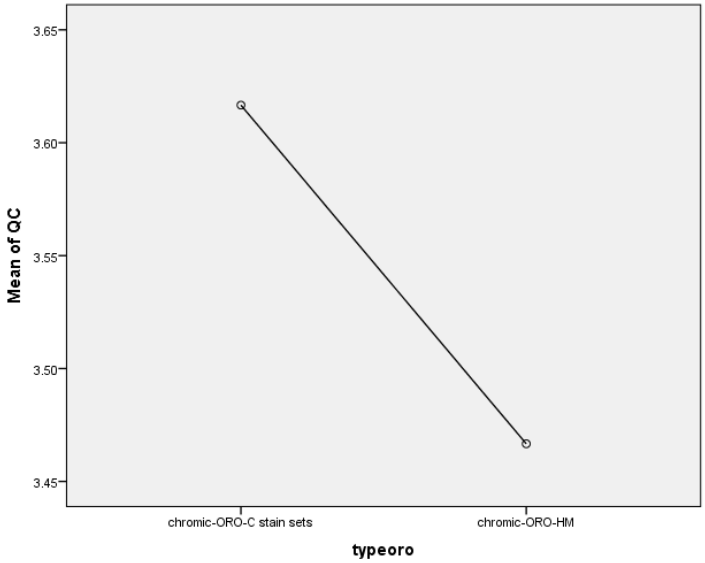


Figure 3. The mean plot of qualitative assessment results between chromic-ORO-C stain sets and chromic-ORO-HM stain in the liver samples

Therefore, the difference in the quality of the two types of lipid dyes, when using lipid fixative tissue with the chromic acid technique, can be elucidated. At the excellent level, chromic-ORO-HM stain showed a lower quality (2.5), compared to chromic-ORO-C stain sets (7.5). However, at the good quality level, chromic-ORO-HM stain showed a higher level (12.0) than the chromic-ORO-C stain sets (6.0). From the results of the statistical analysis, it can be confirmed that there were significant differences in quality between chromic-ORO-C stain sets and chromic-ORO-HM, at the excellent and good levels, as shown in Table 2. This difference may be due to the difference in color shades, where the chromic-ORO-C stain sets produce reddish-orange pigments that are darker than the color chromic-ORO-HM stain for staining fat droplets. However, the chromic-ORO-HM stain provided better background staining, facilitating better readability and interpretation. In other quality levels, the results were not significant different, as shown in Table 1. When comparing experts averages, it was found that the quality of fat staining with chromic-ORO-HM stain quality was not significantly different from chromic-ORO-C stain sets (Table 3 and Figure 3). In addition, it should be noted that neither dyes needed improvement, indicating that the quality of the self-prepared ORO dyes was comparable to that of the commercial dyes and that the chromic acid technique was able to maintain lipid tissue condition (Arregui et al., 2020).

3.3 Assessment of the diagnostic competency of steatosis between ORO-C stain sets and ORO-HM stain by chromic acid technique

The identification of fatty changes on liver steatosis slides stained with chromic-ORO-HM yielded positive findings, ranging from mild to severe, with the mildest yield being 44%. In comparison, the chromic-ORO-C stain sets produced positive results ranging from mild to severe, with the mildest effect being 40%, as presented in Table 4.

In the statistical analysis of the detection of fatty changes in hepatic steatosis stained slides by chromic-ORO-C stain

sets, there was a tendency to report results in descending order: mild, moderate, severe, and not found. For chromic-ORO-HM stain, the tendency to report the results was in descending order: not found, severe, moderate, and mild. Chromic-ORO-C stain sets was most positive with mild (58.7%), while chromic-ORO-HM stain mostly showed not found (61.3%), as shown in Table 5.

The results of the statistical analysis using the chi-square test by SPSS showed that the fatty changes observed with chromic-ORO-C stain sets and chromic-ORO-HM stain in liver steatosis tissues were not significantly different, as shown in Figure 6.

Table 4. Mean±standard deviation of grading steatosis results between ORO-HM stain and ORO-C stain sets on liver tissue slides

Chromic-ORO type	Grading steatosis				Total (n)
	Mild	Moderate	Severe	Not found	
chromic-ORO-C stain sets	12.00±10.53 (40%)	3.67±2.08 (12%)	10.67±11.60 (36%)	3.66±3.05 (12%)	30 (100%)
chromic-ORO-HM stain	13.30±5.77 (44%)	4.67±1.52 (16%)	6.00±5.56 (20%)	6.00±3.61 (20%)	30 (100%)

Table 5. Comparison of levels of diagnostic competency for fatty liver disease between chromic-ORO-HM stain and chromic-ORO-C stain sets

Tissue slides	ORO type		Grading steatosis				
			Mild	Moderate	Severe	Not found	Total
Liver steatosis	Chromic-ORO-C stain sets	Count	44	12	22	12	90
		% Grading steatosis	58.7%	57.1%	41.5%	38.7%	50%
	chromic-ORO-HM stain	Count	31	9	31	19	90
		% Grading steatosis	41.3%	42.9%	58.5%	61.3%	50%

Table 6. Statistical analysis of the correlation between chromic-ORO-HM stain and chromic-ORO-C stain sets

	Value	df	Asymp. sig. (2 sides)
Pearson Chi square	5.791[a]	3	.122
Likelihood ratio	5.825	3	.120
Linear-by-linear association	5.301	1	.021
N of valid cases	180		

Note: chi-squared test, *p*-value <0.05 statistically significant.

3.4 Histopathology feature of liver tissue using H&E stain, chromic-ORO-C stain sets, and chromic-ORO-HM stain

Figure 4 illustrates the histological variations between normal and fatty liver samples using different histological staining methods. H&E staining techniques are effective in distinguishing between normal and diseased livers. Round nuclei and eosinophilic (pink) cytoplasm are characteristics of normal hepatocyte structures, as seen in Figure 4A. Hepatocyte plates spread from the portal vein toward the portal tracts. This differs from Figure 4B, which shows the characteristics of steatosis, macrovesicular fatty changes, or large vacuoles that were not stained. Figure 4C shows the result of the fat staining technique with ORO dye on fresh liver tissue, which was cut by frozen sections. The histopathological features in this image differ from Figures 4D and 4E. Triglycerides are stained in a dark reddish-orange color within the cytoplasm, some cell nuclei are located at the edge of the cells, and the nuclei are not clearly visible. While there was some fat staining in Figures 4D and 4E, which used ORO staining by the embedding section technique, the cytoplasm is not fully stained. However, chromic-ORO-C stain sets can produce a darker reddish-orange color than chromic-ORO-HM stain, but the chromic-ORO-HM stain is more effective in staining nuclei, compared with chromic-ORO-C stain sets.

From all the study data, it is evident that both types of dyes did not show significant difference in detecting positive or non-detectable fat (Table 4 and Table 6). Chromic-ORO-HM stain reported a higher percentage of non-detected fat than chromic-ORO-C stain sets, with 61.3% and 38.5%, respectively. Moreover, it was able to report fat positive results showing the highest at severe level, (58.5%), while the chromic-ORO-C stain sets reported the highest fat positive result at mild level (58.7%) (Table 5). Figure 4 shows the histological results of steatosis from the same autopsy sample using different staining techniques. It was found that staining with H&E could not effectively stain fatty changes, revealing only vacuoles, as lipids are soluble in the processing solvents used for embedding the tissues in paraffin (Figure 4B) (Arregui et al., 2020). At the present, the most commonly used method for staining fat in tissues is frozen sections from fresh tissues, so fatty changes can be clearly detected, as shown in Figure 4C (Milroy and Parai, 2019). When the chromic acid technique was applied to the diagnosis of steatosis, it was found that both the self-prepared ORO and the commercial ORO stains were able to be stained with

fatty changes (Figures 4D and 4E). Although chromic-ORO-HM stained only enables staining of fatty changes to a pale reddish-orange, it provided better background staining, compared to the chromic-ORO-C stain sets, simplifying the detection of fatty changes. It can be seen that the chromic acid technique can effectively preserve lipids in tissue (Arregui et al., 2020) and enhance the capability to diagnose fatty liver disease, compared to an ORO stain from the frozen section. Therefore, the results of this study can be applied in daily forensic science work.

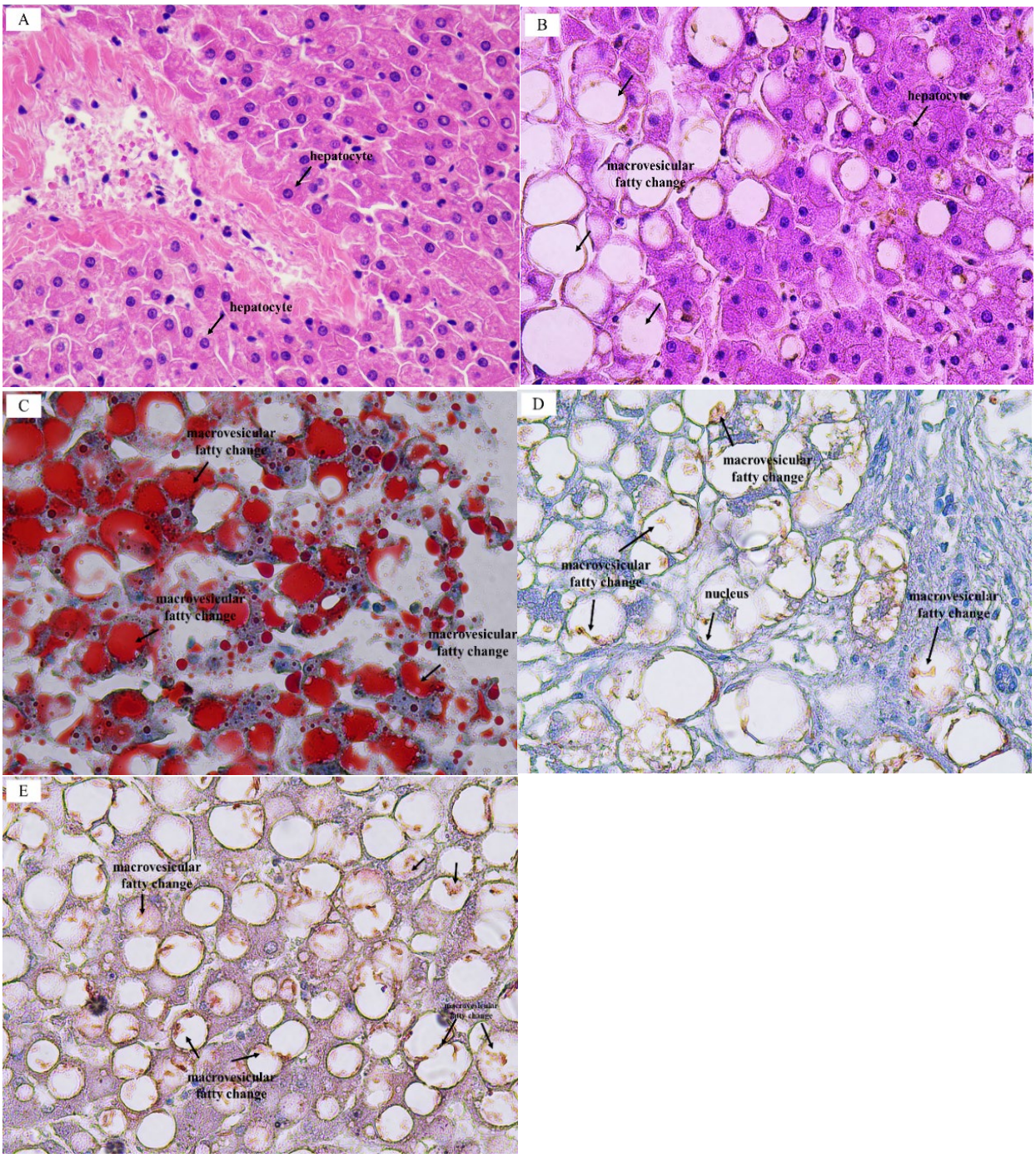


Figure 4. Photographs of liver fatty changes by different staining methods; (A) normal liver by H&E staining, (B) liver steatosis by H&E staining, (C) liver steatosis by ORO by frozen section technique, (D) liver steatosis by ORO-HM stain by the chronic acid technique, and (E) liver steatosis by ORO-C stain sets by the chronic acid technique, 40x magnification.

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

Case1. A 60-year-old male with a history of hospital admission underwent autopsied and examination twice. Organs were injected with formaldehyde for preservation. Relatives suspect that the patient died unexpectedly after receiving treatment.

Autopsy findings revealed no external wounds during the external examination of the corpse. However, internal examination revealed abnormalities in the head, with a significant presence of air in the skull and forehead on both sides. Fractures were observed in the right and left ribs (1 to 7) and the core bone axis between the 1st and 2nd joints. The coronary arteries of the heart had become stiff and thick. Other organs showed traces of formalin fixation. Tissue samples were sent to the laboratory for H&E and ORO staining using chromic acid techniques.

Case2. An 82-year-old female, with a history of vaccination within the past 3 h, experienced abnormal symptoms such as hiccups occurring 2 to 3 times, but she was still able to talk normally. Subsequently, she became unconscious, shaking, and unresponsive. Relatives called an ambulance, but the patient ultimately died at home. The body was delivered for an autopsy due to an unexplained death. Autopsy results revealed no external wounds on the body, no bruises under the scalp, cerebral edema, thickened blood vessels, pulmonary edema, and old myocardial infarction. There were bone fractures of the right and left ribs (2 to 6) and the central bone axis between 2 and 3. Tissue samples were sent to the laboratory for H&E and ORO staining using chromic acid techniques.

Case3. A 29-year-old female, overweight, underwent cause breast augmentation liposuction surgery at a clinic and experienced unnatural death. She was declared dead at the hospital, with a history of resuscitation. The family remained suspicious about the cause of death. Although the first hospital performed an autopsy and found that the cause of death was due to sepsis or septicemia, the relatives requested a second autopsy (Thairath Online, 2020).

During the autopsy, wounds were identified on the upper right arm, left arm, under the left breast, right leg, right leg, and left leg. No abnormalities were discovered during an internal examination of the head, neck, and abdomen. A large bruise wound in the chest extended across the chest area. Tissue samples were sent to a laboratory for H&E and ORO staining using chromic acid techniques.

3.5.1 Findings from autopsies of Cases 1 to 3

In Case 1: Edema in the brain with hypoxic ischemic neurons, old myocardial infarction with acute cell death within 24 h, congested and edematous lungs with blood, bronchioles and alveolar blood congestions, suspected fat embolism in the lungs, and infectious pneumonia.

In Case 2: brain edema, extensive transverse myocardial contraction, congested lungs, and inflammatory cells around the bronchi and pulmonary arteries, suspected fat embolism in the lungs, liver edema, kidney inflammation, and spleen congestion.

In Case 3: Brain edema and partial necrosis, heart edema and congestion, and eosinophil white blood cells in the myocardium, hemorrhage alveoli, fibrosis on the lungs, a small amount of fat and acute nephritis in the liver and pancreas, and no broken ribs

In all three cases, the histopathological results of the lungs with H&E stains (Figure 5) showed rounded, clear holes in the small pulmonary arterial, resembling fat clots in the lungs. Histochemical stains with both chromic-ORO-C and chromic-ORO-HM showed positive fat emboli within the pulmonary capillaries space and small patches along the alveolar wall in all the three cases. Clearly, the reddish-orange color was found. In contrast, the fat droplet stained with the chromic-ORO-C stain sets had a stronger and more pronounced reddish-orange stain dyeing (Figures 5B, 5E, and 5H), compared with chromic-ORO-HM stain (Figures 5C, 5F, and 5I). In addition, we found that the lipid fixative technique well preserved the nuclei. Chromic-ORO-C stain sets had a light orange-gray color background, while chromic-ORO-HM stains had a light grayish-blue background, aiding easier interpretation than chromic-ORO-C stain sets.

Investigators sought autopsies for all three cases to establish the actual cause of death because the death was an unnatural death. From the results of both the external and internal autopsies in all three cases, especially the forensic histopathology laboratory examination using H&E stain techniques, could not explain the cause of death. However, when the pathologist employed the ORO stain, it was found that the cause of mortality could be explained as being caused by fat emboli that detaching and circulating through the pulmonary arteries (Deliliga et al., 2019). The histology indicated spherical, transparent cells that were not stained by H&E, resembling characteristics of fat emboli. The challenge with H&E stains is that the fat is eliminated during the tissue processing, hindering the identification of the death-causing mechanisms.

In general, ORO staining is performed on a frozen section, which the tissue is not processed by a chemical reagent. The researcher was interested in testing using the chromic acid approach, with better preservation of fat in the tissue. As a result, a case study on three victims was conducted. Cases 1 and 2 had a bone fracture while receiving cardiopulmonary resuscitation, leading to death from fat embolism. Case 3 resulted from chest liposuction, causing fat droplets to circulate through the lung arteries. In lung autopsies, performed with the chromic acid technique, revealed positive fat results in both ORO-C stain sets and ORO-HM stain. This indicated that chromic acid technique is an effective technique for determining fat emboli, preserving fat emboli from destruction by xylene (Arregui et al., 2020). In addition, the reddish-orange color of fat emboli can be seen clearly in both dyes.

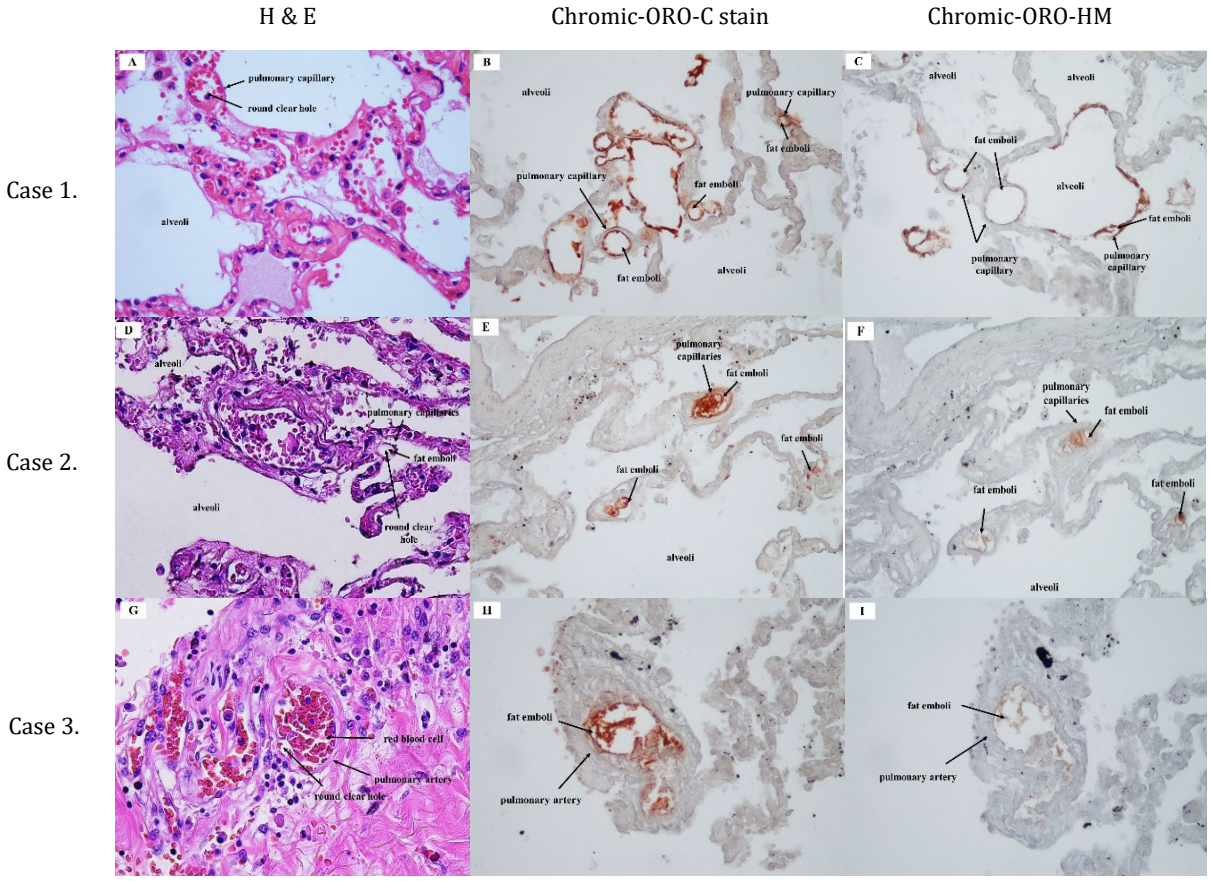


Figure 5. Histopathology features from lung autopsy cases by fat emboli mechanisms with different staining techniques Note: Frames A, D, and G are H&E staining with paraffin sections; the rounded, clear holes seen in the small pulmonary capillaries are characteristics for a fat embolism. Frames B, E, and H are ORO stains from commercial stain sets by chromic acid technique (chromic-ORO-C stain sets), and frames C, F, and I are ORO stains from homemade stains by chromic acid technique (chromic-ORO-HM stain). Fat emboli are within the lung capillaries space (shown with arrows, 40X).

4. CONCLUSION

The study involved a comparative analysis of the efficacy of ORO-HM and ORO-C stain dyes using liver tissue samples with steatosis and normal liver pathology, which characteristics of the samples were confirmed by pathologists. The evaluation included an assessment of the staining quality rated on a 4-scale system: excellent, good, moderate, and needing improvement. It was found that the staining quality of tissue samples post-fixed by the chromic acid technique with ORO-HM stain was not different from that of ORO-C stain sets. However, chromic-ORO-C stain sets had better staining quality than chromic-ORO-HM stain. This superiority lies in the ability of chromic-ORO-C stain sets to produce a darker and clearer reddish-orange stain for fat, while chromic-ORO-HM stain excelled in staining nuclei and providing a clearer contrast between fatty changes and the background, aiding in the detection of fatty alterations facilitating the detection of fatty alterations. The histopathological features of ORO stain in chromic acid-fixed tissue samples supported the findings of the study, indicating the capability of both types of ORO stain in detecting fatty changes in steatosis, not different from ORO stain from frozen sections and H&E stain. The final efficacy assessment of the chromic-ORO-C stain sets and the chromic-ORO-HM stain focused on their ability to report steatosis. It was found that the chromic-ORO-C stain sets detected fatty changes at the severe steatosis level (41.5%) less frequently than the chromic-ORO-HM satin (58.5%). In addition, chromic-ORO-C stain sets reported cases, where fatty changes were not found at 38.7%, while chromic-ORO-HM stain reported such cases at 61.3%. However, the statistical analysis using SPSS revealed that the ability of chromic-ORO-HM stain to diagnose steatosis was not significantly different from that of chromic-ORO-C stain sets. Therefore, when the chromic-ORO-HM stain was used to explain the cause of death in three case studies where the preliminary autopsy results could not determine the actual cause of death by H &E stain alone, histochemical staining techniques can be useful in detecting fat droplets within the pulmonary capillary or alveoli or spreading to different parts of the lung tissue. This technique was able to draw conclusion on the actual causes of death in the three case studies, a fat embolism. Therefore, ORO-HM is as effective as ORO-C stain sets. Moreover, the application of the chromic acid technique to preserve lipid tissue destruction from chemical reagents in the tissue processing procedure can be valuable in elucidating the cause of mortality in cases of unexplained deaths or sudden unexpected deaths.

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