Chula Med J Vol. 45 No. 7 July 2001

Formal saline protocol as an alternative fixation in routine cytological preparation

Kanista Keetacheeva*

Darunee Sachakaew* Pichet Sampatanukul*

Keetacheeva K, Sachakaew D, Sampatanukul P. Formal saline protocol as an alternative fixation in routine cytological preparation. Chula Med J 2001 Jul ;45(7): 569 - 76

Objective : *To compare the quality of smear preparations using fixation with formal saline protocol and fixation with 95 % ethanol*

Design : *Descriptive; questionnaire*

Materials : *Routine specimens submitted for cytopathologic investigation including sputum, bronchial and peritoneal washings and effusions from 20 patients*

Methods : *All samples were prepared in two complementary sets. One set used a formal saline protocol and the other set used routine preparation by wet fixation in 95% ethanol. The quality of background staining and cytomorphologic preservation was evaluated and compared by three reference pathologists.*

Result : *Based on the agreement of at least two referees, better background staining appeared in 12 samples (60 %) in the formal saline fixation protocol, without any worse samples, when compared with ethanol protocol. There were 4 better samples (20 %) but 4 worse samples (20 %) regarding the preservation of cellular details on comparing smears fixed by formal saline protocol and 95 % ethanol.*

* Department of Pathology, Faculty of Medicine, Chulalongkorn University

Conclusion : *Fixation with formal saline protocol was superior to 95 % ethanol with regard to background staining but there was no clear difference between the two protocols regarding preservation of cellular detail.*

Key words : *Formal saline, Fixative, Fixation, Cytological preparation.*

Reprint request : Keetacheeva K, Department of Pathology, Faculty of Medicine, Chulalongkorn University

Received for publication. February 15, 2001.

ขนิษฐา คีตาชีวะ, ดรุณี สัจจาแก้ว, พิเชฐ สัมปทานุกุล. สูตรฟอร์มัลซาไลน์เป็นวิธีทางเลือกสำหรับการรักษาตัวอย่างในการเตรียมทางเซลล์วิทยาตามปกติ. จุฬาลงกรณ์เวชสาร 2544 ก.ค; 45(7): 569 - 76

วัตถุประสงค์ : *เพื่อเปรียบเทียบคุณภาพของการเตรียมสเมียร์เมื่อใช้การรักษาตัวอย่างด้วยสูตรฟอร์มัลซาไลน์ เปรียบเทียบกับ 95 % แอทธานอล*

รูปแบบ : *เชิงพรรณนา, แบบสอบถาม*

วัสดุ : *ตัวอย่างส่งตรวจทางเซลล์วิทยาตามปกติประกอบด้วยเสมหะ น้ำล้างจากท่อหลอดลมและช่องท้องและน้ำท่วมขังในช่องทรวงอกและช่องท้องจากผู้ป่วย จำนวน 20 คน*

วิธีการ : *ตัวอย่างทั้งหมดถูกเตรียมเป็น 2 ชุดควบคู่กัน ชุดหนึ่งเตรียมโดยใช้การรักษา ตัวอย่างด้วยสูตรฟอร์มัลซาไลน์และอีกชุดหนึ่งเตรียมโดยการ รักษาตัวอย่างแบบเปียกด้วย 95 % แอทธานอล คุณภาพของการติดสีของพื้นสเมียร์และการรักษารูปลักษณ์ของเซลล์ ถูกประเมินโดยกรรมการซึ่งเป็นพยาธิแพทย์ 3 ท่าน*

ผล : *โดยใช้เกณฑ์ความเห็นตรงกันของกรรมการอย่างน้อย 2 ท่าน คุณภาพการติดสีของพื้นสเมียร์ดีขึ้นใน 12 ตัวอย่าง (คิดเป็นร้อยละ 60) ในชุดที่เตรียมด้วยวิธีสูตรฟอร์มัลซาไลน์ โดยไม่ปรากฏตัวอย่างที่ด้อยคุณภาพกว่า ตัวอย่าง 4 ตัวอย่าง (20 %) มีคุณภาพที่ดีขึ้นแต่อีก 4 ตัวอย่าง (20 %) มีคุณภาพที่ด้อยกว่าในการเปรียบเทียบคุณภาพการคงรูปลักษณะของเซลล์ เมื่อเปรียบเทียบกันของชุดที่รักษาตัวอย่างด้วยสูตรฟอร์มัลซาไลน์กับชุดที่รักษาตัวอย่างด้วย 95 % แอทธานอล*

สรุปผล : *สูตรฟอร์มัลซาไลน์ มีข้อดีกว่า 95 % แอทธานอลที่ให้ภาพของการติดสีพื้นสเมียร์ที่ดีขึ้น แต่ไม่มีความแตกต่างชัดเจน ในการประเมินคุณภาพของรูปลักษณ์ของเซลล์*

The purpose of a cytological fixative is to maintain as closely as possible the cytomorphologic characteristics and the diagnostically essential cytochemical elements of the cell.[1] Although 95 % ethanol is accepted as universal fixative, compatibility with subsequent immunocytochemical staining may be compromised.[1] The estrogen receptor is lost with alcoholic fixative but can be preserved in a formalin formula.[2] Additionally, in bloodstained smears, blood cells usually cause problems by masking nucleated cells and diminishing the number of cells available for diagnosis. With knowledge of the optimal usage of formal saline in the immunostaining of cell preparations and its property of lysing blood cells,[3] we anticipated the application of formal saline may have advantages in routine cytologic specimen over 95 % ethanol. In order to test the performance of the new preparation, we conducted a study to compare the quality of smears that were prepared by the new fixative protocol with those prepared by the traditional method.

Materials and Methods

Twenty cases of cytological samples that were received at the Department of Pathology, Faculty of Medicine, Chulalongkorn University were used for this study. The specimens included 2 samples of sputum, 5 samples of bronchial washings, 8 samples of pleural effusion, 4 samples of peritoneal washings and one case of ascites. All specimens were freshly received. The fluid samples did not show gross blood staining. The smears were prepared using routine procedures. Sputum was directly smeared on to slides. Centrifugal sediments were collected on slides and direct smearing was applied. Each case had two corresponding sets of smeared slides. One set was processed according to the new protocol of formal saline fixation, as shown in table 1. The other set was wet-fixed by immediate immersion in 95 % ethanol for 15 min. All smears of the two sets were then stained according to the Papanicolaou method.[3]

The two sets of smears were sent blind to three pathologists who were asked to be referees. Each referee examined the same two sets and gave comments on the background staining and cell morphology preservation for each case. Two simple questions were asked to the referees as following, 1. On your consideration, which smear of the pair is better regarding background staining ? and 2. On your consideration, which smear of the pair is better regarding cell morphologic preservation ? There were two choices to each question and each pair of smears of the cases studied; i.e. one was better than the other (which one), or neutral (not different). Comparative superiority of a case was counted when two or three referees showed greater satisfaction with the case in favor of the formal saline fixation protocol. The appreciated background staining is no or less overwhelming blood cells or stark color stain of background that interferes with the microscopic view. The well preservation of cell morphology is no or less shrinkage of cells. The chromatin textures are appreciated.

Results

Table 1 summarizes the two fixation protocols on the main fixative, preparation of fixative and procedure. Table 2 summarizes each referee's opinion regarding the background staining and preservation of cell morphology. Referees' conclusions are present.

Table 1. Preparation of fixatives and fixation protocols.

	Formal saline fixation protocol	Alcohol fixation protocol
1. Main fixative	0.1% formal saline	95 % ethanol
2. Preparation of fixative	Mix 1,000 ml of 0.9 % normal saline and 2.5 ml of 40 % formaldehyde	Commercial or laboratory preparation from absolute ethanol
3. Procedure	Slides allowed to immediately air-dry in room temperature for 10 min before immersing in 0.1 % formal saline for 1 hour and then immersing in 95 % ethanol for another 1 hour.	Immediately immersing slides in 95 % ethanol before cellular smears dry. Slides were in fixative for 15 min.

Table 2. illustration of each referee's opinion and the conclusion on the quality of background staining and preservation of cell morphology.

	BACKGROUND STAINING				PRESERVATION OF CELLS			
Specimen	R1	R2	R3	R-conclusion	R1	R2	R3	R-conclusion
1	B	B	B	BETTER	B	B	B	BETTER
2	B	B	N	BETTER	N	B	B	BETTER
3	B	B	B	BETTER	N	N	N	NOT DIFFERENT
4	B	N	B	BETTER	N	N	N	NOT DIFFERENT
5	B	B	N	BETTER	N	N	N	NOT DIFFERENT
6	B	B	B	BETTER	N	N	B	NOT DIFFERENT
7	B	N	B	BETTER	N	N	N	NOT DIFFERENT
8	B	B	W	BETTER	N	W	N	NOT DIFFERENT
9	N	B	B	BETTER	B	N	N	NOT DIFFERENT
10	B	B	N	BETTER	W	N	B	NOT DIFFERENT
11	N	B	B	BETTER	N	W	B	NOT DIFFERENT
12	B	B	N	BETTER	W	W	W	WORSE
13	B	N	W	NOT DIFFERENT	B	B	B	BETTER
14	B	N	N	NOT DIFFERENT	N	B	B	BETTER
15	N	B	N	NOT DIFFERENT	N	B	N	NOT DIFFERENT
16	N	N	N	NOT DIFFERENT	N	B	N	NOT DIFFERFNT
17	N	B	N	NOT DIFFERENT	N	N	N	NOT DIFFERENT
18	B	N	N	NOT DIFFERENT	W	W	W	WORSE
19	B	N	N	NOT DIFFERENT	W	W	B	WORSE
20	B	N	N	NOT DIFFERENT	N	W	W	WORSE

Abbreviation and Explanation : R1= referee 1; R2= referee 2; R3= referee 3; R-conclusion= the conclusion of referees' opinion; B, BETTER= formal saline protocol gave better result; N, NOT DIFFERENT= both protocols gave no significantly different result; W, WORSE= formal saline protocol gave inferior result.

From the table, it shows the following findings:

1. Background staining was improved in 12 cases (60 %) and not different in 8 cases (40 %) when comparing formal saline protocol with 95 % ethanol. No worse example was revealed.

2. Cell morphology preservation was better in 4 cases (20 %) and worse in 4 cases (40 %) when comparing formal saline protocol with 95 % ethanol. There were 12 cases (60 %) that results from two fixation methods were not different.

Figure 1 depicts one case of effusion which demonstrates the improved background staining seen with the formal saline fixation protocol. Figure 2 illustrates bronchial epithelial cells that are well preserved using both fixation protocols.

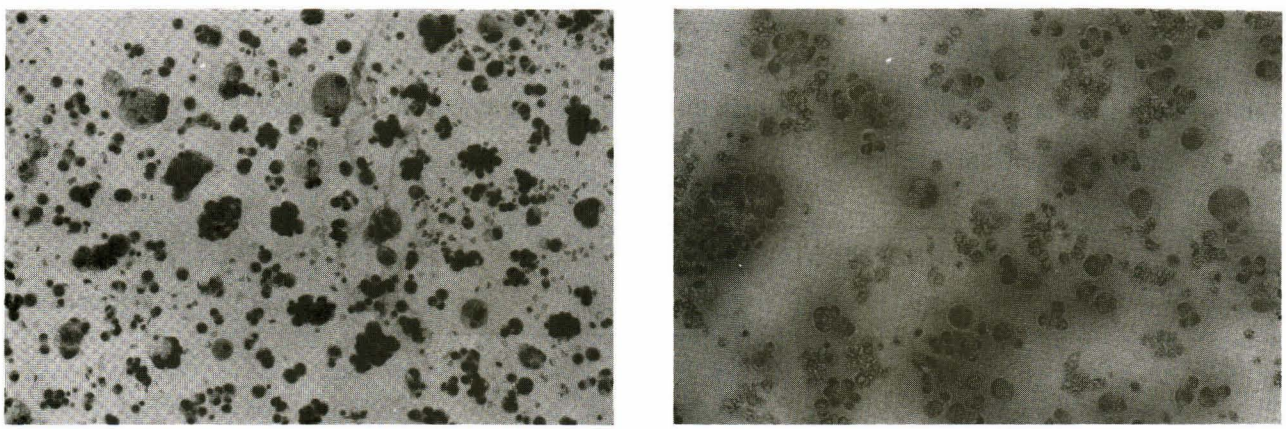

Figure 1. Effusion. The smear prepared from formal saline fixation showed clear background and better contrast than the corresponding smear from ethanol fixation. (A = formal saline fixation smear; B = ethanol fixation smear)

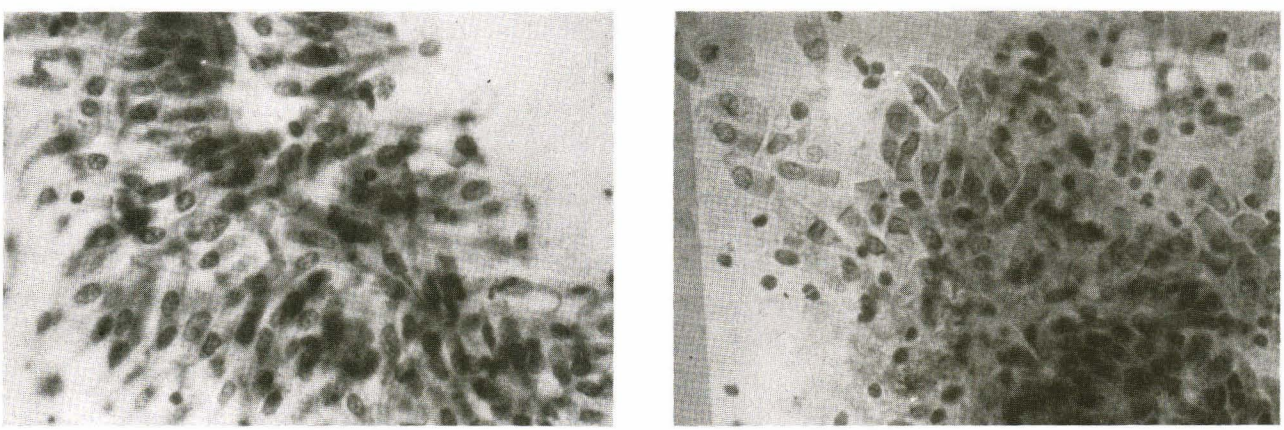

Figure 2. Bronchial washings. The smear prepared with formal saline fixation gave better contrast. The preservation of cell morphology was not different when compared with the smear prepared from ethanol fixation. (A formal saline fixation smear; B = ethanol fixation smear)

Discussion

Fixation is essential to cytology. Cell morphology should preserve as much as possible that of living cell. Until now, 95 % ethanol has been accepted as the universal fixative in cytological preparation, giving excellent nuclear and cytoplasmic details. Formal saline has been known as one of the special-purpose fixatives [3] and its use has been very limited in routine cytological preparation. We had never had experience with this fixative until we became aware that it is the fixative of choice for cytologic preparation for subsequent immunocytochemical process.[2] In order to expand and assess its use, we conducted this research. The results show the value of this fixative for reducing troublesome high background staining. Thus, it will be of benefit for mucus containing and bloody samples. Although the samples we used here were not grossly bloody, they usually contained blood elements in the sediments that were used to smear on the slides. This caused high background staining when processed according to the alcohol fixation protocol. It is therefore useful in this regard to use formal saline protocol to prepare centrifugal sediments. The second point of importance is that these specimens can be also used subsequent immunocytochemical study, if indicated.[4]

There were some arguments regarding the quality of smears in this study. How to assess it ? Since pathologists are responsible for signing out diagnosis, the quality of preparation should be to serve with their contentment and accurate sign-out. The fixatives play some roles on the background staining and good preservation of cellular morphology that provides clear and good contrast of microscopic environment. In this pilot study, we decided to ask the pathologists to express their consideration on the quality of these two aspects. We used the routine cytological specimens in order to determine overview rather specific types of samples. The latter will be the next steps of our study. Bloodstained fluid specimen was not used because this type of fluid needs special care to treat obscuring blood cells.[3] It has been proven from this study that fixation with formal saline protocol can generate clear and less stark background staining. The cost-effectiveness and more work-load are additional issues deserved evaluation that was not performed in this preliminary study. Moreover, preservation of cell morphology involves also factors prior to fixation and processing, i.e. nature and type of sample, collecting method and preservation of fluid specimen.[5] The quality of the cell morphology is therefore difficult to evaluated in details. The conclusion from this study from the experts' opinion is fixation with formal saline protocol and ethanol did not show significant difference.

Acknowledgement

The authors would like to thank Associate Professor Pongsepeera Suwangool, Associate Professor Saowanee Yenrudi and Assistant Professor Voranuch Panyavoravut for kindly being reference pathologists in this study.

References

1. Keebler CM. Cytopreparatoty techniques. In: Bibbo M, ed. Comprehensive Cytopathology, 2nd ed. Philadelphia : W.B. Saunders 889 - 917
2. Suthipintawong C, Leong ASY, Vinyuvat S. Immunostaining of cell preparations: a comparative evaluation of common fixatives

and protocols. Diagn Cytopathol 1996 Aug; 15(2): 167 - 74

3. Proctor DT. Preparatory techniques. In: Coleman DV, Chapman PA, eds. Clinical Cytotechnology. London : Butterworths 1989: 52 - 78

4. พิเชฐ สัมปทานุกุล, พงษ์ศักดิ์ วรรณไกรโรจน์, ปรีชา เรืองเวชวรชัย, มุกดา ตั้งวงศ์ศิริ, ชูศักดิ์ วิรัชชัย. การตรวจตัวรับฮอร์โมนและดัชนีการเพิ่มจำนวนของเซลล์มะเร็งเต้านมโดยวิธีอิมมูนเคมี. จุฬา - ลงกรณ์เวชสาร 2538 พ.ค;39(5): 329 - 35

5. Bales CE, Durfee GR. Cytologic techniques. In: Koss LG ed. Diagnostic Cytology and It's histopathologic bases, 4th ed. Philadelphia: J.B.Lippincott 1992:1451 - 531