Original Article

Effect of Fixatives' Temperatures on Subsequent Histochemical Staining

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ABSTRACT

Background: Fixation is complex series of chemical events which differs for the different group of chemical substances found in tissues. Some chemical reactions, including those involved in fixation occur more rabidly at higher temperature.

Objectives: Toassess the effect of varying fixatives' temperature on the quality of subsequent histochemical staining.

Methods: Rabbit samples were collected including tongue tissue to demonstrate collagen fibers using Van Geison's stain, and liver tissue to demonstrate cell morphology using Erlich's haematoxylin. Specimens were divided into pieces; each sample was fixed in the following fixatives: formal saline, neutral buffer formalin (NBF), Carnoy's and Bouin's fixative in different temperatures as follow 4C°, 25C°, 37C° and 60C°. There after, tissues were embedded in paraffin and cut sections into 5 micron and stained with Ehrlich's hematoxylin and Van Gieson histochemical stains.

Results: For Erlich's heamtoxylin, formal saline gave the best result for tissues fixed at 60C°; NBF gave the best results at 37C° and 60C°. For Van Geison stain, formal saline and NBF the best results obtained at 37 C°.

Conclusion: The study concluded that using 10% NBF, 10% Formal saline, Carnoy's and Bouin's fixatives applying different temperatures include 4C°, 25C°, 37C° and 60C° affect the subsequent histochemical staining of Ehrlich's hematoxylin, and Van Gieson.

Keywords: Fixatives, temperature, Van Geison, Erlich'sheamtoxylin, stain

ixation is complex series of chemical events which differs for the different group of chemical substances found in the tissue[1]. Fixation of tissues can be accomplished by physical and or chemical methods.

Several chemicals and combinations of chemicals can act as good fixatives and accomplished many of stated goals of fixation, some fixatives add covalent reactive groups which may induce cross links between proteins[2]. The best example of such cross-linking fixatives is formaldehyde. Another approach to fixation is the use of agents that remove free water from tissues and hence precipitate and coagulate proteins. Other fixatives such as acetic acid rely on denaturing proteins and nucleic acids through change in pH or via salt formation. Some fixatives are mixtures of reagents and are referred to as compound fixatives. By tradition fixation of surgical specimen is carried out at room temperature, for electron microscopy and some histochemistry the temperature range chosen is 0 – 4 C°. The argument in favors of this lower ranges that autolysis is slowed down, as is diffusion of various cellular components.

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Against this is the fact that the chemical reactions, including those involved in fixation are more rapid athigher temperature[1].The diffusion of molecules increases with rate of penetration of tissue by formaldehyde andis faster at higher temperature. Most chemical reactions also occur more rabidly at higher temperature and therefore formaldehyde reacts more rabidly with proteins[1]. Formalin is an effective fixative that kills most infectious agents in tissue sections, inhibits cellular processes, and preserves tissue architecture[3]. Most laboratories use neutral-buffered formalin (10%) for tissue fixation which introduces cross-links, whereas coagulative fixatives are less popular. Problems with formalin fixation comprise delay of fixation and variations in the duration of the fixation mainly[4].

In this study we tried to detect the effect of four different fixatives namely 10% NBF, 10% Formal saline, Carnoy's and Bouin's fixatives applying different temperatures(4C°, 25C°, 37C° and 60C°)on thehistochemical staining of Ehrlich's hematoxylin, and Van Gieson stains.

Table (1): The relations between the qualities of Ehrlich's hematoxylinand Van Geison stains of tissues fixed by formal saline and NBF at different temperatures.

Fixative &temp	Quality of stain															
	Ehrlich's								Van Geison							
	excellent		Good		Poor		total		excellent		Good		Poor		total	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Formal saline																
4 C°	0	0	10	25	0	.0	10	25.0	0	.0	0	.0	10	25.0	10	25.0
25 C°	0	0	10	25	0	.0	10	25.0	0	.0	0	.0	10	25.0	10	25.0
37 C°	8	20	2	5.0	0	.0	10	25.0	10	25.0	0	.0	0	.0	10	25.0
60 C°	9	22.5	1	2.5	0	.0	10	25.0	9	22.5	1	2.5	0	.0	10	25.0
total	17	42.5	23	57.5	0	.0	40	100	19	27.5	1	2.5	20	50.0	40	100
NBF																
4 C°	0	0	2	5.0	8	20	10	25.0	8	20.0	2	5.0	0	.0	10	25.0
25 C°	0	0	10	25	0	0	10	25.0	9	22.5	1	2.5	0	.0	10	25.0
37 C°	9	22.5	1	2.5	0	0	10	25.0	10	25.0	0	0	0	.0	10	25.0
60 C°	9	22.5	1	2.5	0	0	10	25.0	0	.0	1	2.5	9	22.5	10	25.0
total	18	45.	14	35	8	20	40	100	27	67.5	4	10	9	22.5	40	100

METHODS

A pair of healthy rabbits was scarified, liver and tonguewere selected. The selected organs were cut to one mm thickness pieces of tissue and placed in labeled plastic tissue cassettes.15 blocks were prepared from each organ, one block was prepared from each organ fixed in10% Neutral buffer formalin (NBF), 10% Formal saline, Carnoy's fixative and Bouin's fixative at the applying temperatures of 40^{o}C, 25^{o}C, 37^{o}C and 60^{o}C except Bouin's didn't put in 60^{o}C avoiding it's explosion property.

Fixation time was 18 hour's at 25^{o}C, 37^{o}C and 24hours at 4^{o}C. 6 hours at 60 C° in all fixatives except Carnoy's time which was one hours at 37 C°,and 25^{o}C, 6 hours at 4^{o}C and 30 minute at 60^{o}C. Then dehydrated in 70% and 90% alcohol 3 hours at each change, absolute I and II one hours at each, absolute III and IV two hours at each change, then cleared in xylene I and II two hours at each change and finally

Table (2): The relations between quality of Ehrlich's hematoxylin and Van Geison stain of tissues fixed by Carnoy's and Bouin's fixatives at different temperatures.

Fixative &temp	Quality of stain															
	Ehrlich's								Van Geison							
	excellent		Good		Poor		total		excellent		Good		Poor		total	
Carnoy's	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
4 C°	0	0	1	25.0	1	25.0	1	25.0	1	25.0	0	.0	_	_	1	25.0
25 C°	0	0	1	25.0	1	25.0	1	25.0	1	25.0	0	.0	_	_	1	25.0
37 C°	1	25.0	0	0	0	0	1	25.0	1	25.0	0	.0	_	_	1	25.0
60 C°	1	25.0	0	0	0	0	1	25.0	0	.0	1	25.0	_	_	1	25.0
total	2	50.0	2	50.0	2	50.0	4	100.0	3	75.0	1	25.0	_	_	4	100.0
Bouin's																
4 C°	_	_	1	33.3	0	0	1	33.3	1	33.3	0	.0	_	_	1	33.3
25 C°	_	_	1	33.3	0	0	1	33.3	0	.0	1	33.3	_	_	1	33.3
37 C°	_	_	0	0	1	33.3	1	33.3	1	33.3	0	.0	_	_	1	33.3
60 C°	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
total	_	_	2	66.6	1	33.3	3	100	2	66.7	1	33.3	_	_	3	100

embedded in paraffin wax I and II three hours at each change, except Carnoy's fixed tissue transferred directly to absolute IV and then complete the processing as the other fixatives. Processing and clearing were applied as described by Bancroft and Gamble [1].Then tissue samples were embedded in paraffin standard melting point (52°C–54°C) wax. Tissue was sectioned using rotary microtome. Four micron thickness was cut from each block. Ten sections were obtained from the block that fixed in NBF and formal saline, one section was obtained fromtheblock that fixed in Carnoy's and Bouin's using disposable blade.

Sections were stained by Ehrlich's hematoxylin, and Van Geison stain. All sections were de-waxed and hydrated through graded alcohols to water. Sections of Erlich'shematoxylin were stained in Erlich'shematoxylin for 5-15 minutes), Washed well in running tap water until sections 'blue' for 5 minutes or less, differentiated in 1 percent acid alcohol (1% Hcl in 70% alcohol) for 5-10 seconds, washed well in tap water until section is again 'blue' (10-15 minutes), stained in 1 percent eosin Y for 10 min, washed in running tap water for 1-5 min. dehydrated through alcohols, cleared and mounted[1].

Sections of Van Gieson stain were stained by Celestin blue for 5min, rinsed in distilled water, stained in Mayer's for 5min, differentiated in acid alcohol, washed well in tap water, stainedin Van Gieson solution for 3min, bloted and dehydrated throughalcohols, cleared in xylene and mounted in permanent mounting medium[1].

RESULTS

Table (1) shows the relations between qualities of Ehrlich's hematoxylin and Van Geison stains of tissues fixed in formal saline and NBF at different temperatures.Table (2) shows the relation between qualities of Ehrlich's hematoxylin and van Geison stains of tissues fixed in Carnoy's and Bouin's fixatives at different temperatures.

DISCUSSION

In this study the quality of Erlich's hematoxylin when tissue fixed in formal saline gave the best

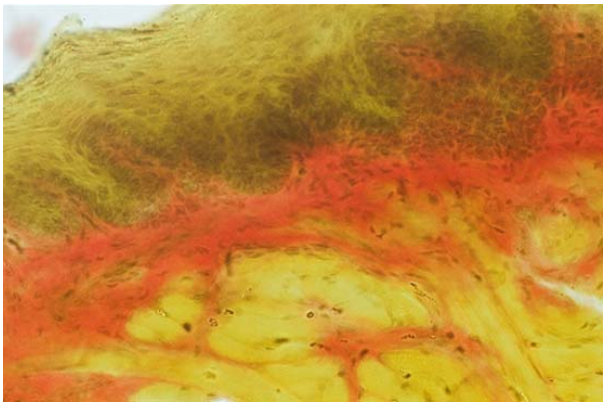

Photo (1): Rabbit's tongue tissue fixed in Carnoy'sshowing excellent collagen fibers [VanGeison stain at 37°C (x40)]

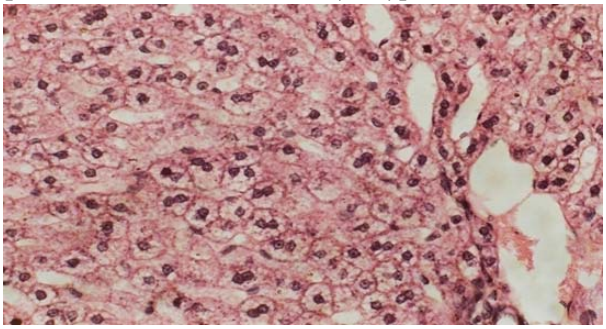

Photo (2): Rabbit's liver tissue fixed in NBF showing excellent tissue preservation [Ehrlich's stain at 60°C (x40)]

results at 60˚C and gave better results at 37˚C. This is attributed to the fact that fixation is more rapid at higher temperature[5]. This study showed that the quality of Ehrlich's hematoxylin when tissue in fixed in NBF gave best results at 37C° and 60C°, and obtained better results at 25C° but at 4C° the results were fair. These results are in agreement with Bussolati et al who found no distinction was observed in morphology between tissues processed either using standard formalin fixation at room temperature or cold fixation (4C°) methods[6]. The present study found no significant association between the qualities of Ehrlich's hematoxylin stain when altering the temperatures of Carnoy's or Bouin's fixative. In this study quality of Van Geison stain of tissues fixed in formal saline fixative at different temperatures showed best results at 37C° and 60C°, and fair result at 4C° and 25C°. Wang[7] observe collagen fibers fixed with polyepoxy, the overall fixation rate was found to be reaction rate controlled, as might be expected. The reaction rate was favored by a higher temperature, concentration and solution pH. His finding explains present study finding. Quality of Van Gieson stain when fixed in neutral buffer formalin showed best results at 37C°, 25C° and 4C°and fair results obtained at 60C°.This study showed no significant association between quality of van Gieson stain and Cranoy's and Bouin's fixatives when fixed at different temperatures.

CONCLUSION

The study concluded that using 10% NBF, 10% Formal saline, Carnoy's and Bouin's fixatives applying different temperatures include 4C°, 25C°, 37C° and 60C° affect the subsequent histochemical staining of Ehrlich's hematoxylin, and Van Gieson. In Erlich'sheamtoxylin, formal saline gave the best result for tissues fixed at 37C° and 60C°, while in Van Geison stain, formal saline and NBF gave the best results at 37 C°.

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