Volume 2 (2), 2014, Page-131-137
e-ISSN: 2348-6465

Pharma Health Sciences

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Effect of delay of fixation on mitotic counts in histopathological sections of tumors

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ARTICLE INFO

Received: 09 Mar 2014
Accepted: 20 Apr 2014

ABSTRACT

Mitotic count is the most commonly used method of assessing the proliferative activity of a tumor. Mitotic count should be done very meticulously because it is used to evaluate prognosis of various tumors and is one of the cornerstones for the diagnosis and grading of malignant tumors. *Aims and objectives*: Factors affecting mitotic count starts from the initial stage of fixation itself. There are numerous other variables that can also influence the mitotic count; our study is designed to assess effects of delay of fixation on the mitotic counts. *Materials and methods*: We studied 40 cases which composed of 30 cases of Infiltrative duct carcinoma, Breast & 10 cases of high grade Non Hodgkin's lymphoma. To study the effect of delay in fixation. Comparisons of mitotic count/ High power field made between sections fixed immediately, after 1 hour and after 6 hours. The correlations between mitotic counts and Ki-67 labeling indices were also looked *Results:* our study showed that lack of prompt fixation ($p<0.001$) led to significant change in mitotic counts. Both mitotic counts and ki-67 index were significantly higher in immediately fixed specimens compared to those fixed after 1 hour. There was only weak correlation between Ki-67 index and mitotic count. *Conclusion:* Better precision can be obtained in mitotic counts by prompt fixation of the surgical specimens. This can be achieved by good coordination between surgical and pathology teams.

Key words: Mitosis, fixation, delay

1. INTRODUCTION

Markers of proliferation have been extensively investigated to evaluate prognosis of various tumors and is one of the cornerstones for the diagnosis and grading of malignant tumors.

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The mitotic count is the most commonly used method of assessing the proliferative activity of a tumor. Other newer methods to detect the proliferative fraction of tumor cells include immunohistochemical detection of Ki67/MIB-1, DNA flow cytometry and measurement of S –phase fraction. But all these methods are complicated and time consuming. Commonly in our routine precise we do make an assessment of proliferative activity of tumor by counting the mitotic figures and in special circumstances we opt for KI67/MIB-1 index which is a direct indicator of the growth fraction. The mitotic counts thus obtained are used for classification, grading, predicting prognosis of tumors and sometimes even advocated as a decision point for treatment.[1,2]

Since the proliferative activity of the tumor as assessed by mitotic counts are very important in many aspects of diagnosis and treatment we should be very cautious and vigilant while counting them in H&E slides. There are numerous variables that can influence the mitotic counts. Starting with .first and the early phase of tissue processing, fixation[3] .Delay of fixation has profound effects on mitotic counts. In one study, a delay of 60 & 180 minutes before fixation, mitotic counts was observed to be reduced to 49.4% & 15% respectively.[3] Other factors resulting in variable counts include thickness of the section [4], size of the high power field of the microscopes, failure to locate the mitotically active area of the tumor, too few or too many fields counted and so on. Also apoptotic cells and neutrophilic granulocytes can mimic mitotic figures[1]. So it is very important to stick to correct morphological criteria and a strict counting protocol to get reproducible mitotic counts.

This study is designed to assess the relative importance of delay of fixation affecting the mitotic counts, the field which got relatively little attention previously, and to suggest ways to overcome them. Also we tried to correlate mitotic counts obtained after various time delays with proportion of proliferating cells as estimated by MIB-1 immuno histochemistry

1.1 Effects of delay of fixation on mitotic counts

Fixation is the first and most essential step in tissue preparation for microscopic analysis. A well fixed tissue is the key for a good slide and so for a good diagnostic interpretation. This step helps to stabilize tissue proteins and to prevent autolysis and putrefaction. Fixation preserves tissue integrity as well as protects antigenicity. Routinely used fixative is 10% formalin which is a cross linking fixative. Tissues should be placed in fixative as soon as they are removed from the body. But usually there will be a delay of 15-40 minutes before fixation. Delay of fixation can have different effects on mitotic counts in different tissues as shown by previous studies

Fixation delay can have different effect on mitotic counts in normal or malignant tissue. While the study on normal human cervical epithelium found no difference in the numbers of mitotic figures in those which were fixed immediately and in those which were incubated at 37'C for up to two hours [5], those in normal colonic mucosa declined by about 30% with a delay in fixation of two hours and by 50% with a delay of six hours. [6]

R.D Start et al studied the effect of delay in fixation on the modified Bloom and Richardson grade of 8 breast carcinoma cases. Each tumor samples were immersed in fixative at times of 0.5, 2, 4, 6, 18 and 24 h after surgical removal. A delays of 0.5 and 6 hours resulted in a decrease in grade of the tumors which was primarily due to reduced number of mitotic figures which was seen to be declined by a mean of 53%. [7]

Most of the studies regarding the effect of fixation delay were conducted on small number of samples which resulted in selection bias. Researches based on larger number of specimens (604 samples) showed that

within the time investigated, delay of fixation has no clear influence on the proliferation features. The reduced counts resulted from poorer morphology in the slides, causing more difficult identification of mitotic figures. This interpretation was strengthened by flowcytometric measurements. [8]

Estrogen receptor (ER) is a strong predictor of response to hormonal treatments in patients with invasive breast cancer. So an accurate and standardized detection of ER expression is essential to proper and appropriate clinical management. Fixation problems can also result in erroneous interpretation of ER also.[9]

Not only in breast carcinomas, is the effect of delayed fixation evident on soft tissue sarcomas and in Non Hodgkin's lymphomas also. [10] A study by Graem et al on human osteogenic sarcoma showed almost rapid decline in the number of mitotic figure even for a minimal delay of three hours. They also recorded the number of mitotic figures in different phase of mitosis. They concluded that there is a relative accumulation of the advanced phases with increased delay, since only those cells which are already entered the mitotic phase will complete that mitosis. And very few cells will enter the mitotic phase after removal of tissue. [3]

The apparent differences in previous studies conducted in this field prompted us to do a newer study to find out the relative importance of time lag before fixation on mitotic counts in our clinical and laboratory settings.

1.2 Ki-67 -- Mitotic marker

Cell-proliferation markers are very important in the clinical management of cancer patients especially in the carcinomas of the breast. Identification of Ki-67 protein coded by the MK167 gene made it easier to define the level of proliferative activity. MIB-1 is the most commonly used monoclonal antibody which detects the Ki-67 antigen on the formalin-fixed paraffin-embedded tissue sections which is known as Ki-67 labeling index.

It is a nuclear protein which is necessary for cellular proliferation. It is also associated with transcription of ribosomal RNA. During mitosis most of the antigen is relocated on to the chromosomal surface, but during interphase it is detected exclusively within the nucleus. Ki-67 antigen is present during G_1, S, G_2, and mitosis phase of cell cycle, but is absent in G_0.

MIB1 labeling index shows strong association with histological grade of the tumor, tumor size and tumor type .The results also suggest that the tumor growth fraction, as assessed by the MIB1 labeling index, is an important predictor of survival. [11] In another study a significant association was found between Ki-67 values and tumor size, nodal status, estrogen and progesterone receptor status. Multivariate analysis showed that Ki-67 levels were associated with disease-free and overall survival, thus confirming that it is an independent prognostic variable. [12]

1.3 Objectives

- **T**o delineate the relative contribution of delay in fixation leading to variability in mitosis counting.
- To estimate the correlation of mitotic counts obtained after different times before fixation with proportion of proliferating cells as estimated by MIB-1 immuno histochemistry.
- To formulate guidelines to reduce the effect of time delay on mitotic counts and to achieve uniformity in mitosis counts.

2. MATERIALS AND METHODS

Study conducted in the department of Pathology, Medical College, Kozhikode, Kerala during the academic year of 2010-2011. Our study sample composed of 30 cases of Infiltrative duct carcinoma, Breast & 10 cases of high grade Non Hodgkin's lymphoma. We selected two different types of tumors with high proliferative activity where mitotic counts

play a key role in therapeutic management. And by combining two different samples effects of biological variation can also be reduced to some extent.

The counts made by the principal investigator on 4-5 micrometer thick sections on a Labomed microscope in sections fixed 1 hour after removal are considered as the standard. For estimating the effect of delay in fixation all specimens were collected from the operation theatre immediately after removal. They were cut and examined. One bit from the tumor immediately immersed in 10% formalin. Another bit of the same size kept apart for fixation after 6 hours. The rest of the specimen immersed in fixative after 1 hour and sent for routine grossing. Comparisons of mitotic count were made between sections fixed immediately, after 1 hour and after 6 hours. The proliferating cells were labeled by MIB-1 antibody and a Polymer-HRP IHC Detection System (Biogenex). The results expressed as percentage of positive tumor cells. Data storage and analysis were done with EPIINFO software

Ethical issue: Consent taken for surgery. No other ethical issue involved.

3. RESULTS

The main results are presented in the table. There is a progressive decline in mitotic counts as the time to fixation is prolonged. (Figure 1, Table 1)) There is progressive decline in the proportion of Ki-67 positive cells also (Figure 1) The mitotic counts obtained by various fixation intervals showed strong correlation (Table 2**).** There is a significant correlation between mitotic counts and Ki-67 index in tissues fixed immediately but not for the others (Table 3).

4. DISCUSSION

Total number of cases studied was 40, which included 30 cases of infiltrating duct carcinoma breast and 10 cases of high grade Non Hodgkin's lymphoma. In carcinoma breast 70% of the cases belonged to grade 2, 10% to grade 1 and 20% grade 3 categories. All Non Hodgkin lymphoma studied were high grade.

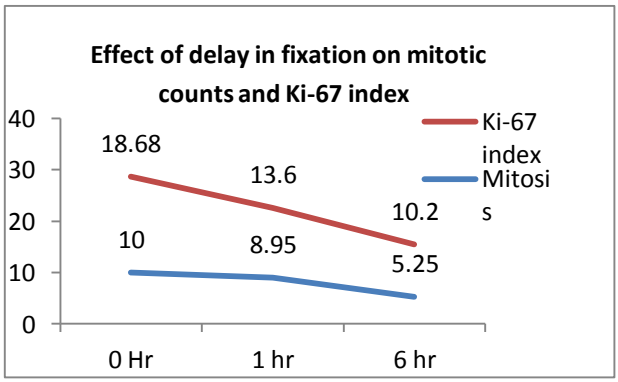

Fig 1: Effect of delay in fixation on mitotic counts and Ki-67 index.

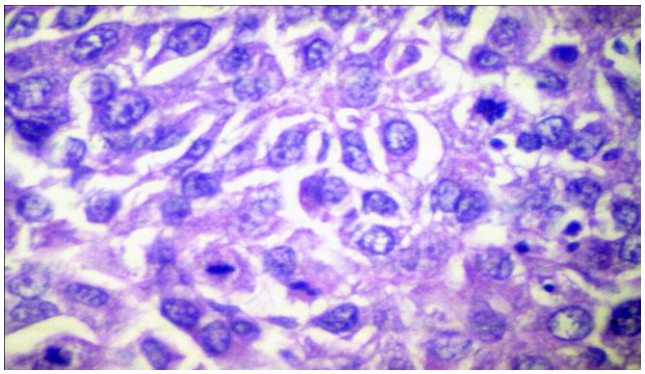

Fig 2: Mitotic figures in a high grade breast carcinoma. Note the clear or lightly stained cytoplasm of mitotic cells. H&E x 400.

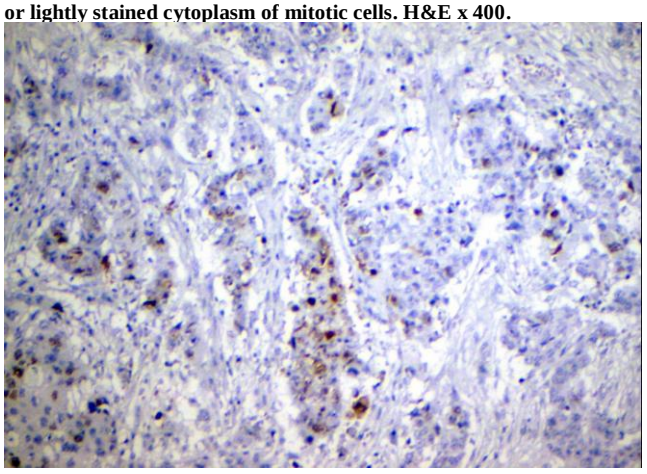

Fig 3: Nuclei of tumor cells labeled with antibody to Ki-67.

Among the 30 cases of breast cancer, there was one case of metaplastic carcinoma and one apocrine carcinoma. All the rest were infiltrating duct carcinomas - not otherwise specified. Among the lymphomas there were 7 diffuse large cell lymphomas (DLBCL) and 3 anaplastic large cell lymphomas (ALCL).

Counting mitosis and expressing it as a quantitative figure per a set number of high per fields is a time

honored method used by histopathologists in the assessment of cell proliferation. Though extremely common in practice, this method has been criticized for its imprecision or lack of reproducibility. Many of the factors associated with method, instrument and observer have in turn been blamed for this variation. In this study we have tried to delineate the effect of fixation delay, one of the most of significant of these factors that lead to lack of precision, so that simple guidelines can be formulated to achieve acceptable levels of uniformity.

Table 1: Comparison between mitotic counts obtained in sets of data in paired parameters

Parameter 1	Mean (95% CI)	Parameter 2	Mean (95% CI)	Paired t test	
				t	p
1 hr after fixation	8.95(8.1 - 9.8)	Immediate fixation	10.0 (9.1-10.9)	3.1	0.003
1 hr after fixation	8.95(8.1 - 9.8)	6 hr after fixation	5.25 (47.8-9.6)	13.8	<0.001

Table 2: Correlation of standard mitotic counts with counts under different circumstances

Parameter	Correlation coeffiecient (R)	F	p
Immediate fixation	0.711	38.8	<0.0001
6 hr after fixation	0.799	67.2	<0.0001

Table 3: Correlation of standard mitotic counts with Ki-67 indices at different times of fixation

Parameter	Correlation coeffiecient (R)	F	p
Ki-67 at 0 hr	0.324	4.5	0.041
Ki-67 at 1 hr	0.294	3.6	0.065
Ki-67 at 6 hr	0.199	1.6	0.218

Sections from thirty cases of breast cancer and ten cases of high grade lymphoma formed the study material. A mixture of two types of lesions was selected to reduce chances of systematic error and biological variation. Mitotic counts done by the principal investigator on the sections fixed after 1hour is taken as the standard. The mitotic counts obtained at 0 hour and 6 hour was compared with the standard. Paired t test and linear regression were the statistical tests used in analysis.

Fixation was done for each specimen by three alternative methods. The specimen was received immediately after removal in the operation theatre and cut. A piece of the tumor was fixed immediately (0 hrs); another piece was fixed after 6 hours and the main tumor fixed after 1 hour. The period of 1 hour was considered the standard, since it usually took this much time to fix the specimen in the theatre before being sent at leisure to the pathology department. Frequently in practice, however, the addition of formalin takes much longer. It is for measuring this effect that a piece fixed at 6 hours was chosen.

Figure 1 show that there is a progressive decline in mitotic counts as the time to fixation is prolonged. The fall is sharp from 1 hour to 6 hours (Table 1). But even at one hour there is a significant decline in mitotic counts as compared to immediate fixation. Right from the time of removal, cells in the mitotic phase progress along the cycle and some of them complete the process by the time the tissue is fixed. Figure 1 also shows the progressive decline in the proportion of Ki-67 positive cells. Here the fall between immediate fixation and fixation after 1 hour is sharper than in the case of mitotic counts. The conclusion seems to be inescapable that immediate fixation of tumor tissue should be the norm if correct mitotic counts and Ki-67 index are to be obtained. In cases like breast cancer and soft tissue sarcomas, where mitosis forms an important grading parameter, prompt fixation can to be ensured by proper co-ordination with the operation theatre staff.The mitotic counts obtained by various fixation intervals showed strong correlation, even though as seen earlier, the means differed significantly. This shows that a systematic error is occurring, uniformly acting on all cases producing lower or higher counts (Table 2).

The correlations between mitotic counts and Ki-67 labeling indices were also looked for. There is a significant correlation in tissues fixed immediately but not for the others. Even for the former the correlation co-efficient is not very strong (R= 0.324; p=0.041). (Table 3) Indeed, this is not surprising since mitosis

and Ki-67 labeling look at biologically different group of cells. Ki-67 labels all cells in the non G0 phases of the cell cycle, while mitotic figures represent the G2-M phases only.[13] This underlies the fact that mitotic counts cannot be replaced by proliferative indices and that both are better used in a complementary fashion.

5. CONCLUSION

Our study showed that lack of prompt fixation ($p<0.001$) led to significant change in mitotic counts. Both mitotic counts and ki-67 index were significantly higher in immediately fixed specimens compared to those fixed after 1 hour. There was only weak correlation between Ki-67 index and mitotic count. Better precision can be obtained in mitotic counts by prompt fixation of the surgical specimens. This can be achieved by good coordination between surgical and pathology teams.

6. ACKNOWLEDGEMENT

It is a great pleasure to express my gratitude and indebtedness to my guide **Dr. Sathi P P** (Professor and Head, Department of Pathology, Govt. Medical College, Kozhikode) for her guidance, encouragement, moral support and motherly affection throughout the period of my work. I would like to place my gratitude to **Dr Joji Maliekkal** (Professor, Department of Surgery, Govt. Medical College, Kozhikode) without whose help I wouldn't be able to do this study.

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