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Pathology

The Numerical and Morphological Changes in Cytology of Pleural Fluids after Prolonged Storage at Room Temperature

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Abstract **Original Research Article**

Aim & objectives: 1) To evaluate the TLC, DLC in pleural fluid on day 0,1,3 & 5 by manual and automated methods and also the cytomorphological changes and compare the changes over time. 2) To compare the TLC between manual and automated method. ***Methodology***: 36 samples of pleural fluid were included and evaluated within 4 hrs on day 0 and analysed again on 1st, 3rd, and 5th day after storage. TLC was done using improved Neubauer chamber as well as automated hematology analyser. The DLC was done in stained smears and the morphological changes were also noted. ***Rsults:*** Both the manual and the automated analyser showed a significant reduction of TLC over 5 days with p values of 0.161 and 0.006 respectively (ANOVA). Also change in neutrophil lymphocyte ratio was observed with a significant reduction in the neutrophils ($p<.05$) and increase in lymphocyte ($p<.05$) over 5 days. There was positive correlation between the TLC in manual and automated method with a highly significant p values (<.001). The first morphological changes noted were vacuolization with cell swelling, loss of lobular structure and cytoplasmic rim and nuclear changes were other changes. ***Conclusion:*** The pleural fluid samples should be processed timely (preferably within 4 hrs) for accurate results, however, under unavoidable circumstances can be delayed till 24 hrs without many changes. The TLC and DLC is not reliable in the fluid processed >24 hrs and almost 90% cells are degenerated by day 5.The automated and manual methods are comparable.

Keywords: TLC, DLC, pleural fluid.

INTRODUCTION

Pleural effusions are a common medical problem and may suggest a pulmonary, pleural and extrapulmonary disease [1]. Thoracocentesis is a low cost procedure and is a reliable method for the diagnosis of cause of pleural effusion. The lab analysis includes the White Blood Cell (WBC) count and the differential counts [2]. The proper collection and handling of the samples is of utmost importance as it may have a direct impact on the results [3]. A WBC count of >1000 cells /uL and <1000 cells/uL suggests exudate and transudate respectively [4].

Pleural fluid with neutrophil predominance indicates an acute inflammatory process and lymphocytes indicate a subacute or chronic process [2]. The increased lymphocytes in pleural effusions are commonly seen in tubercular and malignant effusions but can also be seen in rheumatoid disease, lymphoma, sarcoidosis, and chylothorax [1]. Mesothelial cells are a normal part of the pleural effusions [4].

All the fluid samples should be examined promptly for reliable interpretation of the sample and diagnostic accuracy. The cellular elements are expected to lyse with time and the fluids with large number of erythrocytes deteriorate faster than clear fluids and leukocytes begin to deteriorate as early as 30 minutes of collection.[2] The type of container as well as the presence or absence of anticoagulant also influences the counts as there may be clot formation without anticoagulant leading to decreased counts.[2] According to literature there is little data regarding the changes in the WBC counts as well as cytomorphological changes in the pleural fluid samples over time[2,4].

Therefore, we take this study with an aim to find the difference in the counts as well as the morphological changes which appear with time when the samples are stored at room temperature.

METHODOLOGY

The study was a prospective study and was

conducted in the Department of Pathology, ESIC Medical College and Hospital within a period of two months (July- Aug 2018). Samples of pleural fluid were evaluated within 4 hrs on day 0 and analysed again on 1st, 3rd , and 5th day after storage at room temperature(20-22 degC) in anticoagulant (ethylenediaminetetraacetic acid)tube. The TLC was done using improved Neubauer chamber as well as automated hematology analyser. The cytocentrifuged samples were stained with May-Grunwaldt Giemsa stain and differential counts obtained as well and the changes in morphology of different cells were also noted on 0, 1st, 3rd, and 5th day.

Statistical analysis

The one way ANOVA test was used for evaluation of difference in the TLC (both by manual and automated methods) and DLC in the pleural fluid samples stored over a period of 5 days. Perason's coefficient (r) was calculated to evaluate the correlation between the manual and automated methods for TLC. A p value <.05 was considered statistically significant.

RESULTS

A total of 36 samples were included in the study over a period of 2 months. Age of the patients included in the study ranged from 14 years to 72 years. Mean age of the patients was around 46 years. There were 23 males and 13 females in the study. M: F ratio was 1.76:1.

Total leucocyte count was done using both manual (improved neubaur chamber) and automated method on day 0,1,3,5. There was a reduction of TLC on succeeding days by both the methods as elicited by table 1 and 2 (Total mean by manual and automated method was 1606.4583 and 1333.2639 with standard deviation of 2439.2202 and 1563.2291 respectively). The decrease in TLC on succeeding days was statistically significant with p value <0.05 by both manual and automated methods.

The TLC by manual and automated methods yielded similar results on all the successive days with significant correlation with a p value <.00001 which was highly significant (Table 5). (Correlation coefficient (r) of correlation between the TLC by manual and automated method on day 0,1,3,5 is 0.781, 0.673, 0.770, 0.811 respectively). The TLC by manual and automated method is illustrated in fig 2.

Differential leucocyte count and morphological changes were assessed on day 0,1,3,5 using giemsa stained smear. There was a decrease in neutrophil count and increase in lymphocyte count on succeeding days as elicited in table 3 and 4 (Mean neutrophil and lymphocyte count was 22.146 and 77.84 with standard deviation of 25.414 and 25.4079 respectively). This was also statistically significant shown by a p value <0.05. The variations in the neutrophils and lymphocytes in the samples stored for 5 days is illustrated in fig 3.

Mainly six morphological changes were observed in the stained smear on succeeding days:

- Cell swelling
- Vacuolization/loss of granules
- Loss of structure of lobe
- Nuclear changes
- Change in cytoplasmic rim
- Change in chromatin.

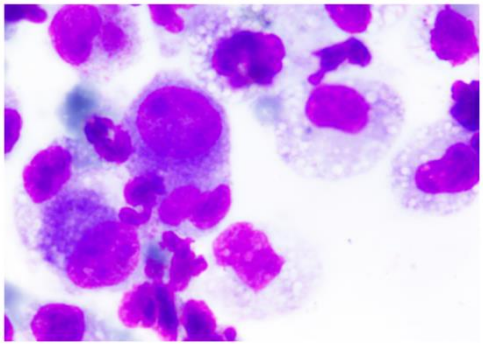

Fig-1: Giemsa stained smears show cellular swelling and vacuaolization.(1000X, day 3)

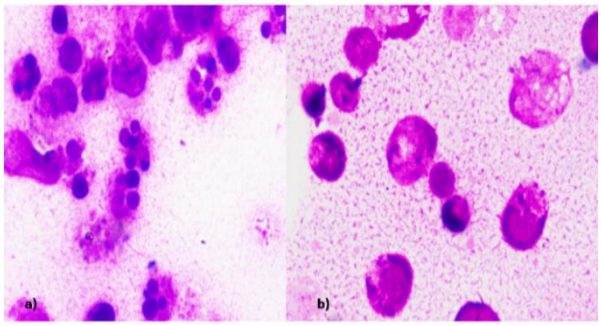

Fig-2: a) Giemsa stained smears show degenerating neutrophils with loss of lobular structure. (1000X, day 5). b) Giemsa stained smears show complete cellular degeneration.(1000X, day 5)

Table-1: Variation of TLC with manual method (improved neubauer chamber) after storage

Day	0	1	3	5	Total	P value
N	36	36	36	36	144	0.016
∑X	92950	63380	43600	31400	231330	
Mean	2581.9444	1760.5556	1211.1111	872.2222	1606.4583	
Std.Dev.	2784.4464	2923.3884	1940.5489	1589.2246	2439.2202	

Table-2: Variation of TLC with automated hematology analyser after storage

Day	0	1	3	5	Total	P value
N	36	36	36	36	144	0.006
∑X	77150	50000	39790	25050	191990	
Mean	2143.0556	1388.8889	1105.2778	695.8333	1333.2639	
Std.Dev.	2115.9649	1464.1971	1215.8042	857.7691	1563.2291	

Table-3: A Correlation of TLC done by manual method and automated hematology analyser

Day	Manual metod(mean TLC)	Automated method(mean TLC)	Correlation coefficient(r)	p value
0	2581.94	2143.05	0.781	<0.00001(HS)
1	1760.55	1388.88	0.673	<0.00001(HS)
3	1211.11	1105.27	0.770	<0.00001(HS)
5	872.22	695.83	0.811	<0.00001(HS)

Table-4: Variation in neutrophils (derived from DLC) after storage

Day	0	1	3	5	Total	p value
N	36	36	36	36	144	**0.007**
∑X	1005	905	670	609	3189	
Mean	27.9167	25.1389	18.6111	16.9167	22.146	
Std.Dev.	29.3349	27.1105	19.9145	23.713	25.414	

Table-5: Variation in Lymphocytes (derived from DLC) after storage

Day	0	1	3	5	Total	p value
N	36	36	36	36	144	0.007
∑X	2593	2695	2930	2991	11209	
Mean	72.0278	74.8611	81.3889	83.0833	77.84	
Std.Dev.	29.3019	27.1105	19.9145	23.713	25.4079	

DISCUSSION

Pleural effusions constitute a significant cause of morbidity [1]. Due to the varied causes of pleural effusion a systemic approach is required to arrive at an accurate diagnosis [1]. Thoracocentesis, which is a safe and low cost procedure, is the initial step in the diagnosis of cause of pleural effusion [2]. The reliability and accuracy of the pleural fluid analysis may be greatly affected by improper collection and handling of the specimen [3]. The fluids should be collected in EDTA or heparin treated tubes to avoid clotting and cell clumping for biochemical, microbiological and

cytological analyses [3]. Blood culture bottles are used for microbiological analyses [3]. The routine analysis of pleural fluids is done for protein, LDH, gram stain, AFB stain, cytology and microbiological culture [1]. The appearance and odor of the pleural fluid is of high significance and should always be recorded [1].

The present study was conducted with the aim of assessing and documenting the changes in the pleural fluid analysis when it is stored for prolonged periods. For reliable and accurate results, all the pleural fluid samples should be sent to the laboratory as soon as possible to prevent the disruption of cellular morphology and disappearance of the cells [2]. One of the study has reported that both temperature and storage time are a cause of error in the evaluation of pleural fluid cytology with decreased counts as well as morphological alterations [5]. The fluid with large number of erythrocytes shows more rapid cell degeneration than the clear fluids. Additionally if the fluid is sent without anticoagulant in a glass or plastic tube may also result in false counts due to clot formation [6].

In our study the samples were stored at room temperature in EDTA tubes over a period of 5 days and manual and automated counts were done on 0,1st,3rd and 5th day. We found a significant reduction in the TLC by both manual and automated methods. Garlipp et al had also demonstrated a significant reduction in the TLC on storage for 72 hrs, however, the TLC though reduced after 24 hrs, was not statistically significant which differed with our study where a significant reduction of TLC was seen even after 24 hrs[2].

Garlipp *et al*. also reported a constant reduction in the percentage of neutrophils and an elevation of lymphocytes in the pleural fluid samples upon storage, the result however, was not statistically significant [2]. In our study the reduction of neutrophils and elevation of lymphocytes in the pleural fluid samples on storage was found to be statistically significant. Guzman et al demonstarted that there was a marked decrease in the lymphocyte subpopulation which were attached to the slide by peroxidase-antiperoxidase assay when it was stored for four days at room temperature, however not much reduction in the count was seen after 24 hrs[7]. The above findings reinforce the importance the timely processing of the pleural fluid samples. The maximum acceptable time for delay before processing of pleural fluid samples is 2 hrs according to literature.[8] It has been recommended by some authors that for future research studies the fluid sample be centrifuged and supernatant or pellet be stored at -80 degree celcius[3].

We studied the morphological changes in cell cytology on giemsa stained smears and found that cell morphology was relatively preserved after 24 hrs, however on 3rd day the cytoplasmic changes mainly vacuolization and cell swelling were the first changes noted. The nuclear changes were maximally apparent by day 5 where there was complete nuclear degeneration. Manosca *et al*. had demonstrated that the cytomorpholology of the fresh effusion samples were preserved for upto 14 days if the samples are refrigerated at 4 degree celcius[9]. Other authors have also stated that morphology is preserved for a period of 24-72 hrs if samples are stored under appropriate refrigerated conditions [3].

We found that there was an excellent correlation in the TLC between manual and automated methods on all the successive days and the result was highly significant with a p value <.001. It has been reported by various authors that the counts by automated and manual methods had comparable results when the samples were collected in EDTA tubes. The WBC counts varied when the sample was collected in the glass or plastic tube without anticoagulation [4,10].

Manual counting is a labor intensive, time consuming process and is also dependent on the availability of skilled laboratory personnel. There might be also sampling error due to relatively small number of cells counted manually, at least 1000 cells to be counted in order to arrive at a precise result. The modern automated counters are capable of analyzing 1,000 to 20,000 leukocytes from each sample in an hour; thereby reducing the hours of labor utilized [11]. Thus, automated counters can be used reliably for the TLC of the pleural fluid samples.

CONCLUSION

Pleural effusion is a common medical problem suggesting pulmonary, pleural and extrapulmonary disease. There was a significant decrease in TLC after 24 hrs by both manual and automated method on prolonged storage at room temperature. Neutrophils showed significant decrease and relative increase in the percentage of lymphocytes. Cytological changes like vacuolization, cell swelling appears after 72 hrs while nuclear changes appeared on day 5 with complete nuclear degeneration. Automated and manual count shows excellent correlation. Thus, the pleural fluid should be analysed as early as possible preferably within 4 hrs and to be stored under appropriate conditions if the delays are expected.

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Ethical statement

The ethical clearance was taken from the institutional ethical committee.

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