DOI: 10.7860/NJLM/2024/59835.2824

Original Article

Pathology Section

Use of Absolute Alcohol in Preservation of Cell Morphology in Fluid Cytology at Various Temperatures: A Cross-sectional Study

NEEMA TIWARI[1], NAVYA JAISWAL[2], ARCHITA KANSAL TIWARI[3], RANI BANSAL[4], SHIVANI TOMAR[5]

ABSTRACT

Introduction: Fluid cytology and the examination of malignant cells play an important role in the treatment of critical cases. The Department of Pathology (Cytopathology laboratory) receives samples for fluid cytology and examines malignant cells, reporting the White Blood Cell (WBC) count in the received fluid and the morphological findings of the cells examined on smears. Degeneration of cells in these body fluid samples, especially if the samples are not stored under proper conditions, is a common problem faced by many laboratories. Even overnight storage (12 hours) of these body fluids at temperatures of 4-8ºC (domestic refrigerators) may still cause some degree of degenerative changes. This becomes problematic in cytopathological reporting of body fluids, particularly when the samples are limited in amount or are from areas that cannot be re-aspirated {e.g., Cerebrospinal Fluid (CSF)}. While alcohol is a common fixative used for cell fixation in cytopathology, it is rarely used for fixing cells in fluid samples.

Aim: To test the usage of absolute alcohol as a fixative in body fluid samples received in the cytopathology laboratory, which have been stored for 12 hours at two temperatures: room temperature and 4-8ºC.

Materials and Methods: A cross-sectional study was conducted in the Department of Pathology at Subharti Medical College, Meerut, Uttar Pradesh, India. The study was conducted over the periods of two months, from February 2022 to March 2022. A total of 100 body fluid samples (including CSF, ascitic fluid, pleural fluids, etc.) sent for routine workup to the cytopathology laboratory were included in the study. Each body fluid sample (1 mL) was taken in two test tubes, and four drops of 100% (absolute) alcohol were added to both test tubes. The mixture of body fluid and absolute alcohol was then gently shaken, and one set of test tubes was kept at room temperature while the other set was kept at 4-8ºC. The data was tabulated in an MS Excel worksheet, and basic statistical analysis was performed.

Results: The age range of cases whose samples were analysed was from one-week-old neonates to 65-year-old adults. The total number of fluid samples was 100, including 42 CSF samples, 23 ascitic fluid samples, 18 pleural fluid samples, and 17 Bronchoalveolar Lavage (BAL) fluid samples. Out of the 42 CSF samples, 15 showed preservation of morphology when the alcohol-body fluid mixture was kept at room temperature, while 22 cases showed preservation of the same mixture at 4-8ºC. Some samples showed no preservation (5 CSF), the reasons for which are discussed below. All the examined body fluids showed better preservation with alcohol at 4-8ºC.

Conclusion: The addition of absolute alcohol as a fixative in fluids helps preserve the cytomorphological features of cells and provides better preservation for the diagnosis of fluid samples.

Keywords: Cytomorphological features, Degenerative changes, Malignant cells, White blood cells

INTRODUCTION

Fluid cytology is an integral part of cytopathology, where excess fluid that accumulates in various body cavities (e.g., pleural cavity, peritoneal cavity, and pericardial cavity) is aspirated and sent to the Pathology Department for examination and diagnosis. Fluid cytology and the examination of malignant cells play a crucial role in the treatment of critical cases. Fluid cytology is used to diagnose malignant cytology in fluids and for emergency cell counts. It provides a quick and simple way to diagnose benign and even malignant lesions (such as blast counts in acute leukaemias in the case of CSF), enabling clinicians to receive a probable diagnosis in a short period of time [1].

The fluid is usually received in the laboratory after being stored at room temperature in the wards and Outpatient Departments (OPD) or transported to the laboratory without ice packs. In some cases, the transit time is significantly prolonged, especially in hospitals located in villages and peripheral areas. This time delay between obtaining a fresh fluid sample and examining it under a microscope can lead to poor or inaccurate results due to cell degeneration [1]. Accurate reporting can help confirm the diagnosis and determine the appropriate course of management, greatly benefiting the patient. However, obtaining fresh samples for degenerated fluid samples, such as CSF or pericardial fluid, is not always feasible. Therefore, it is crucial to properly preserve these samples, as prolonged transport time and improper storage are common issues in laboratories, particularly in hospitals located in peripheral areas.

The present study was conducted in a Tertiary Care Centre that caters to a large population and receives samples not only from in-house hospitals but also from affiliated hospitals. The authors from the Department of Pathology aimed to develop a simple, inexpensive, and cost-effective method to extend the preservation time of cell morphology in fluid samples for upto 12 hours for cytology and malignant cell reporting. Although the use of alcohol as a preservative is known, especially in Papanicolaou smears and liquid-based cytology, the novelty of this work lies in the fact that, to the best of the authors' knowledge, there are no published studies where alcohol drops have been used in fluid samples received in cytopathology for the preservation of cell morphology. Present study was aimed to investigate the use of alcohol as a preservative by mixing it with a defined quantity of body fluids and also, to compare the benefits of storing the mixture of alcohol and sample (alcohol+sample) at overnight temperatures of 4-8ºC (domestic refrigerator door) with those stored at room temperature, in order to extend the preservation time from two hours to 12 hours.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Department of at Subharti Medical College, Meerut, Uttar Pradesh, India. The study was conducted over the periods of two months, from February 2022 to March 2022. A total of 100 fluid cytology specimens were analysed. Ethical clearance was obtained by the Institutional Ethics Committee (IEC) with reference number SMC/IEC-2022-012.

Inclusion criteria: The inclusion criteria consisted of fresh samples of fluids and CSF received in the emergency Department of Pathology (clinical pathology). Only samples sent for routine processing were included in the study. No special sampling was done solely for the purpose of the study.

Exclusion criteria: The exclusion criteria included old degenerated samples and samples received in extremely small quantities that were insufficient for the study.

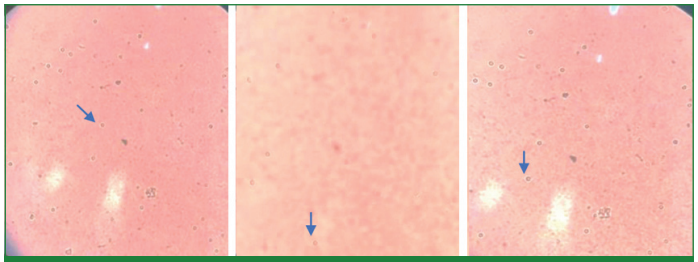
Study Procedure

The samples were divided equally into two glass test tubes for each body fluid examined. Four drops of absolute alcohol were added to each test tube, and one test tube containing the alcohol plus body fluid mixture was kept at room temperature, while the other test tube with the same mixture was kept at 4-8ºC [Table/Fig-1a,b].

[Table/Fig-1]: Sets of fluids examined were prepared in two test tube racks one containing fluid plus alcohol mixture left to stand at room temperature (a); and another set of test tubes with alcohol plus sample was kept in the fridge (b). (Images from left to right)

The samples were kept at the aforementioned temperatures for 12 hours, and a comparison of microscopic cell morphology was performed. Observational data was collected and charted. The observed percentages were subjectively calculated by the pathologists, who counted 10 fields (total number of cells, both degenerated and preserved) and then counted the preserved cells in all those 10 fields to calculate a rough percentage. For example, if the total number of cells preserved plus degenerated in 10 fields (denominator) is X and the total number of viable cells in 10 fields (numerator) is Y, the calculation formula is Y/X×100. For instance, if Y=8 and X=12, the preserved cellularity would be calculated as follows: Answer- 8/12×100=66.7%, showing preserved morphology.

In a few samples, centrifuged stained deposits [Table/Fig-2a-c] of the alcohol-sample mixture were prepared to confirm the present study's findings recorded from examining the unstained slides. The stains used to stain the smears were Leishman and Giemsa stains.



[Table/Fig-2]: a) CSF showing 60% cellularity in fluid examined after 12 hours of refrigeration containing four drops of absolute alcohol (arrow, 10x); b) unstained drop preparation of fluid-alcohol mixture kept at room temperature for 12 hours showing approx. 40% cellularity (arrow, 10x); c) another fluid plus alcohol mixture was kept in the refrigerator for 12 hours showing almost 50% preservation of cell morphology on examination of unstained drop preparation (arrow,10x). (Images from left to right)

STATISTICAL ANALYSIS

The data was tabulated in an MS Excel worksheet, and basic statistical analysis, such as calculating the mean, was performed.

RESULTS

A total of 100 cases were included in the study, with 57 males and 43 females, resulting in a male-to-female ratio of 1.3:1. Out of the 57 males, 17 samples were from the paediatric age group (<18 years), and out of the 43 females, 29 samples belonged to the paediatric age group. In total, 46 samples were from the paediatric age group, with 20 cases from the neonatal Intensive Care Unit (ICU) and the remaining from the emergency ward and Pediatric Intensive Care Unit (PICU). The age range of cases whose samples were analysed was from one-week-old neonates to 65-year-old adults. For the remaining 54 adults, samples received were from the emergency ward, neurosurgery ICU, medicine wards, ICU, Critical Care Unit (CCU), and surgery ward. Out of the total 100 body fluid samples taken, 42 were CSF samples, 23 were ascitic fluid, 18 were pleural fluid, and 17 were BAL fluids [Table/Fig-3]. Out of the 42 CSF samples, 15 showed preservation in morphology when the alcohol and body fluid mixture was kept at room temperature, while 22 cases showed preservation of the same mixture at 4-8ºC. Some samples showed no preservation (5 CSF), the reasons for which are discussed below. Out of the 23 ascitic fluid samples (alcohol fluid mixture), nine showed preservation at room temperature, 11 showed preservation at 4-8ºC, and three showed no preservation. All the body fluids examined showed better preservation with alcohol at 4-8ºC [Table/Fig-4].

Sample type	Number of cases (n)	Request on the requisition form by the clinician
CSF	42	Cytology (in one sample from this 42 clinicians wanted to analyse for any blasts in CSF apart from the routine cytological workup for TLC and DLC)
Ascitic fluid	23	Cytology (TLC/DLC) and cytology for malignant cells
Pleural fluid	18	Cytology (TLC/DLC) and malignant cell
BAL fluid	17	Cytology for malignant cell with few cases also requiring TLC/DLC values with malignant cell cytology

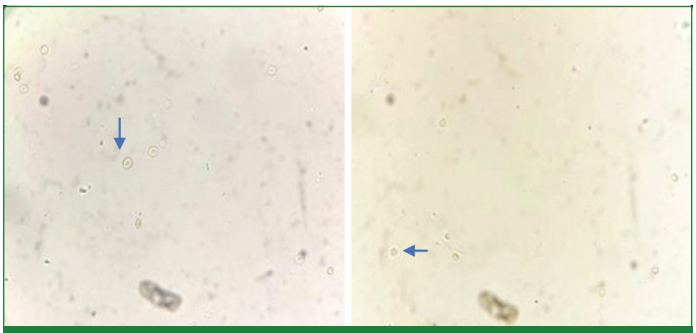
[Table/Fig-3]: Types of samples received and investigation required (N=100).
CSF: Cerebrospinal fluid; TLC: Total leukocyte count; DLC: Differential leukocyte count

Fluid type	Number of samples alcohol mixture and mean percentage of cells preserved room temperature after 12 hours	Number of samples showing preserved cell morphology with added alcohol at kept in the refrigerator (4-8ºC) after 12 hours	No-preservation of cell morphology after 12 hours in samples with alcohol
CSF (total 42 cases)	15 cases (38% preserved morphology)	22 mean percentage (57% preserved morphology)	5 (3 room temperature+2 in fridge)
Ascitic fluid (total 23 cases)	9 (25% preserved morphology)	11 (60% preserved morphology)	3
Pleural fluid (total 18 cases)	6 (17 % preserved morphology)	11 (70% preserved morphology)	1
BAL fluid (total 17 cases)	6 (40% preserved morphology)	10 (60% preserved morphology)	1

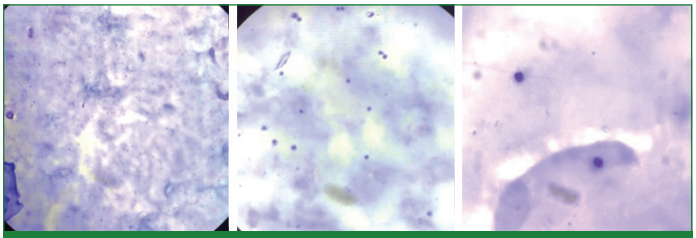
[Table/Fig-4]: A comparative analysis (observational) of preservation of morphology in different fluids over a period of 12 hours at room temperature versus 4-8ºC.

Microscopic examination of the slides prepared from the fluid-alcohol mixture showed better preservation of cell morphology at 4-8ºC than at room temperature [Table/Fig-4]. [Table/Fig-2a-c] (left to right-2a-c] show cells preserved in CSF after 12 hours where the mixture is kept at 4-8ºC [Table/Fig-2a,c], and at room temperature [Table/Fig-2b]. It is evident that samples kept at four degrees show better preservation of cell morphology than samples kept at room

temperature. [Table/Fig-5] shows similar findings in ascitic fluid. Stained smears were also made [Table/Fig-6a-c] using Leishman-Giemsa stain for some cases to confirm findings of unstained smears. The reason for some samples in each category showing no preservation despite using alcohol is discussed later.



[Table/Fig-5]: Unstained smears of ascitic fluid preserved with alcohol in refrigerator (left, 5a, 10X) and at room temperature (right, 5b, 10X) showing preserved morphology at room temperature (arrows).



[Table/Fig-6]: Stained deposits of fluids with alcohol kept in refrigeration showing preserved cell morphology at (10X, 40X, 100X; left to right, respectively).

DISCUSSION

Normally, the serous cavities are lined by monolayered mesothelial cells and contain a minimal amount of fluid to aid in lubrication. In various neoplastic and non neoplastic conditions, serous effusions may develop in the serosal cavities like pleural, peritoneal, and pericardial. The diagnosis in such conditions as inflammatory, autoimmune, and infectious aetiology can be made by studying the exfoliated and exudated cells. The most common sample in the cytology laboratory comprises serous effusions and pelvic washes. Fluids like pleural are either aspirated with a wide-bore needle inserted into the body cavity through the body wall or collected intraoperatively, e.g., pelvic and peritoneal wash by flushing the peritoneal sac with a balanced salt solution and collecting it. The correct method of collection, storage, and processing can help in accurate and definitive reporting of the sample versus that of an uninterpretable sample [2,3].

A well-preserved fluid sample can provide relevant diagnostic information, making cell morphology preservation crucial. Often, the Pathology Department (specifically the cytopathology laboratory) receives samples for fluid cytology, including malignant cells. In the present study, authors reported the cell count of white blood cells (and red blood cells) in the received fluid and describe morphological changes observed on smears. These samples are frequently received at night. However, for fluids received specifically for malignant cells or cytology, a second opinion from experienced senior pathologists may be required. Hence, the fluid needs to be preserved for some time, potentially upto 12 hours. Unfortunately, it has been observed that even if the fluid is kept at a temperature of 4-8ºC during this period, the cells start to degenerate, resulting in the loss of valuable cytological material for diagnosis. This degradation can be a significant problem as fluid samples are often available in small amounts and sometimes can only be aspirated once (e.g., CSF).

In a review article or study on preanalytical errors in fluid samples, a literature search was conducted using the Ovid Medline and PubMed search engines. This search yielded a total of 659 records on the topic. The report emphasised the importance of providing appropriate training to clinicians regarding fluid collection and explaining the need for early transport to improve sample viability. For cytological analysis and subsequent triage for additional testing, body fluids are best examined in their natural state. However, practical issues make it necessary to consider a cheap and easily available preservative for fresh fluid samples that cannot be examined immediately. This need for a preservative is essential to increase diagnostic sensitivity [4].

In line with other studies, it is recommended to have a minimum of 100 mL of ascitic fluid for proper cytopathological processing and evaluation [5]. Studies conducted on the minimum adequacy volume required for cytological evaluation have suggested that 60 mL and 75 mL are the minimum volumes of pericardial and pleural fluids, respectively, needed for adequate processing. For all practical purposes, CSF can typically be collected in 8-10 drops, which is less than 1 mL. However, occasionally we receive CSF samples up to 1 to 1.5 mL, depending on the clinician performing the lumbar puncture. In the present study only four drops of alcohol per mL of fluid were used, which is a small and cost-effective quantity [5,6]. The properties of certain body fluids, such as higher concentrations of fibrin or thrombin, require specific collection methods. These fluids have the potential to form clots and coagulate. Therefore, samples for such fluids are collected in heparinised vials/containers or syringes containing 3 units of heparin per 1 mL bottle capacity [1,2].

While collection techniques like heparin can affect findings, such as causing lower pH, no documented side effects were observed after adding absolute alcohol to these samples. However, it is important to note that biochemical parameters could have been altered. To mitigate this, biochemistry labs receive a separate container of fluid for biochemical analysis, and the alcohol-mixed fluid is used only in the Pathology department [7]. Body fluids in different body cavities are rich in proteins, so it is better to analyse fresh fluids, which spread better and are better preserved compared to older fluids where protein denaturation has occurred [1]. Refrigeration of fresh fluids at 4ºC is recommended by many. In a study conducted by Guzman J et al., they examined cell number, morphology, and immunocytochemical staining with HEA-125 from day 0 to day 4 in fluids collected in Ethylenediaminetetraacetic Acid (EDTA)-coated tubes and stored at either 4°C or room temperature. They observed that immunocytochemical staining remained consistent until day 4, although some lymphocytic markers were negatively affected by storage [8]. Manosca F et al., studied batches of fluids refrigerated at 4°C from day 0 to day 14 and concluded that morphology, immunocytochemical profiles, and amplifiable Deoxyribonucleic Acid (DNA) were all preserved [9]. Antonangelo L et al., observed the effect of storage at either 4°C or -20°C on the levels of adenosine deaminase in effusions suspected to be of tubercular origin. They concluded that the enzyme levels could be measured in samples stored at 4ºC or -20ºC for up to 28 days without significant changes [10].

In the present study, two preservation temperatures were tested using the same sample-to-preservative ratio: room temperature and refrigeration at 4-8ºC. As mentioned in the above-quoted studies [10], lower temperatures result in less impairment of enzymatic activity within cell cytoplasm. This suggests that lower temperatures enhance cell preservation and delay degeneration. Ideally, fresh samples are preferred for evaluation, but in rare cases where samples need to be shipped to a centralised laboratory, preservation may be necessary. However, none of the previous studies used alcohol as a preservative, which the authors did. By using alcohol in combination with refrigeration, the authors were able to significantly preserve the cell morphology of the fluid sample even after 12 hours. However, some samples in the present study showed no preservation of morphology despite the addition of alcohol. The authors hypothesise that the following reasons may have contributed to this:

- The sample was sent to the laboratory 24 hours after withdrawal.

- Incorrect sampling was performed by a resident with minimal or no expertise.
- The sample taken out in time was not stored properly in the ward.
- The sample was not transported properly, maintaining the temperature chain.

There are papers that discuss the use of Balanced Electrolyte Solution (BES), also known as a physiological solution, for lavages. BES is sterile and pyrogen-free, making it suitable for in-vivo or in-vitro use. However, it is more expensive, sold in small amounts, and is typically only required for cell cultures [1]. There is a single study that discusses the usage of commercially available alcohol-based fixatives, such as CytoLyt™ (methanol-based media used for ThinPrep™) and CytoRich™ (ethanol-based media for SurePath), which can be added to an equal volume of the sample. Other alcohol-based preservatives like saccomanno can also be used [11]. However, their availability is limited to larger centers and is mostly applicable to cervical smears. The cost per case is high in such scenarios, and consistent results may not always be guaranteed. The method in the present study, on the other hand, is safe, applicable to all fluids (even those requiring air-dried smears), does not require mechanical processors, and produces good results.

Once the specimen is received in the laboratory, it is entered into the LIS system and assigned a laboratory number. The volume and gross characteristics of the fluid received are evaluated and documented, including factors such as colour, clarity, and any unusual features like viscosity. The easiest, most efficient, and cost-effective method for fluid examination is the evaluation of a wet drop sediment using a coverslip. Many laboratories use machines such as cytospins, Millipore filters, and LBP, as well as Cell Block (CB), to concentrate the fluid and increase cell yield. The preferred staining method for these slides is the Romanowsky stain or the Papanicolaou method after ethanol fixation. The present study technique is applicable to all these types of samples [12,13].

Limitation(s)

The study has definite limitations, as there were only a small number of samples in each category that did not show any preservation even after the addition of alcohol and following the described method. There could be various causes for this, such as samples being left unattended in wards for extended periods before being sent to us, or faulty collection methods leading to cell damage. Additionally, samples sent from bags (like ICD tubes, for example, pleural fluid) were not mentioned in the requisition forms from multiple hospitals. Moreover, a larger sample size and a more precisely developed scoring system would be required to definitively prove the role of alcohol as a preservative for emergency fluids.

CONCLUSION(S)

In conclusion, using absolute alcohol as a preservative for body fluids is an efficient method for cell preservation. It is easily accessible as a laboratory reagent and can be handled easily by trained or untrained staff. In the present study, this preservation method yielded good results in maintaining cell morphology at lower temperatures. Not only was this preservation method effective for all types of samples received in the laboratory, but it is also more cost-effective compared to reagents used in techniques like thin prep and Surepath, which may not be available in every laboratory.

REFERENCES

[1] Gill GW. Cytopreparation: Principles & practice, Rosenthal D, editor. London: Springer, 2013.
[2] Roffe BD, Wagner FH, Derewicz HJ, Gill GW. Heparinized bottles for the collection of body cavity fluids in cytopathology. Am J Hosp Pharm. 1979;36(2):211-14.
[3] Naylor B. Pleural, peritoneal and pericardial fluids. In: Boibbo M, editor. Comprehensive cytopathology. Philadelphia: WB Saunders, 2008:515-77.
[4] Michael WC, Davidson B. Pre-analytical issues in effusion cytology. Pleura and Peritoneum. 2016;1(1):45-56.
[5] Rooper LM, Ali SZ, Olson MTA. Minimum volume of more than 60 mL is necessary for adequate cytologic diagnosis of malignant pericardial effusions. Am J Clin Path. 2016;145(1):101-06.
[6] Rooper LM, Ali SZ, Olson MT. A minimum fluid volume of 75 mL is needed to ensure adequacy in a pleural effusion: A retrospective analysis of 2540 cases. Cancer Cytopathol. 2014;122(9):657-65.
[7] Guzman J, Arbogast S, Bross KJ, Finke R, Costabel U. Effect of storage time of pleural effusions on immunocytochemical cell surface analysis of tumor cells. Anal Quant Cytol Histol. 1992;14(3):203-09.
[8] Guzman J, Arbogast S, Bross KJ, Finke R, Costabel U. Effect of storage time on the analysis of lymphocyte subpopulations in pleural effusions. Acta Cytol. 1993;37(3):267-71.
[9] Manosca F, Schinstine M, Fetsch PA, Sorbara L, Maria Wilder A, Brosky K, et al. Diagnostic effects of prolonged storage on fresh effusion samples. Diagn Cytopathol. 2007;35(1):06-11.
[10] Antonangelo L, Vargas FS, Almeida LP, Acencio MM, Gomes FD, Sales RK, et al. Influence of storage time and temperature on pleural fluid adenosine deaminase determination. Respirology. 2006;11(4):488-92.
[11] Kapse SS, Arakeri SU, Yerranguntla DP. Rehydration of air-dried smears with normal saline: An alternative for conventional wet fixation method in cervical cytological study. J Cytol. 2018;35(4):199-203. Doi: 10.4103/JOC.JOC_186_17. PMID: 30498289; PMCID: PMC6210821.
[12] Rupinder K, Shubra W, Kanwal M. Rehydration of air-dried smears versus wet fixation: A Cross-sectional study. Acta Cytol. 2013;57(4):364-68.
[13] Elgert PA, Gill GW, George N. Papanicolaou. Cytopathology Lab Med. 2009;40(4):245-46.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Pathology, School of Medical Science and Research, Sharda University, Greater Noida, Uttar Pradesh, India.
2. Assistant Professor, Department of Pathology, Subharti Medical College, Swami Vivekanad University, Meerut, Uttar Pradesh, India.
3. Assistant Professor, Department of Pathology, Subharti Medical College, Swami Vivekanad University, Meerut, Uttar Pradesh, India.
4. Professor, Department of Pathology, Subharti Medical College, Swami Vivekanad University, Meerut, Uttar Pradesh, India.
5. Resident, Department of Pathology, Subharti Medical College, Swami Vivekanad University, Meerut, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Neema Tiwari,
School of Medical Science and Research, Plot No. 32, Knowledge Park 3,
Sharda University, Greater Noida-201310, Uttar Pradesh, India.
E-mail: nehaneemat@yahoo.co.in

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Sep 05, 2022
- Manual Googling: Mar 17, 2023
- iThenticate Software: Jul 19, 2023 (10%)

ETYMOLOGY: Author Origin

EMENDATIONS: 10

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Aug 24, 2022**
Date of Peer Review: **Feb 20, 2023**
Date of Acceptance: **Jul 20, 2023**
Date of Publishing: **Apr 01, 2024**