Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (3), 2024; 790-794

Evaluation of Turmeric extract for human spermatozoa morphology assessment in combination with haematoxylin

Research Article

Rubina MP[1*], Sukesh[2], Soumya V[1], Mohammed Safeer TK[1], Riyas Basheer KB[3]

1. Research Scholar, Allied Health Sciences, 2. Professor, Department of Pathology,
Srinivas Institute of Medical Sciences & Research centre, Mukka, Mangaluru, Karnataka. India.
3. Associate Professor, Department of Physiotherapy, Tejasvini Physiotherapy College, Kudupu, Mangalore, Karnataka. India.

Abstract

Introduction: This study aims to assess the effectiveness of utilising Turmeric extract as a sustainable alternative to synthetic Eosin dye in the commonly employed Haematoxylin and Eosin (H and E) staining method for analysing human spermatozoa morphology. While H and E staining yields high-quality results, the synthetic nature of Eosin raises concerns about its environmental impact. By exploring the use of Turmeric extract as a substitute, this research aims to provide a more eco-friendly solution without compromising the staining quality. Method: A solution of Turmeric extract, comprising 15 grams per 100 ml, is formulated using 70% alcohol. This alcoholic extract is substituted for Eosin in the process of H and E staining. In the experiments, two sets of semen smears are prepared. One set is subjected to H and E staining, while the other set undergoes staining with Haematoxylin and Turmeric (H and T). The quality of staining on each slide is assessed based on various parameters, including the background of the smear, as well as the distinct sections of the sperm, namely the head, neck, body, and tail. Result and conclusion: Turmeric confers a unique coloration to the stained spermatozoa, yielding a vivid and distinguishable visual effect that contrasts with the conventional pink staining achieved through the use of Eosin. In both staining techniques, the morphology of the spermatozoa is clearly observable and distinguishable, albeit with Eosin providing enhanced contrast and sharpness. The exceptional staining capacity of Turmeric paves the path for the exploration of natural alternatives to supplant synthetic dyes.

Keywords: H and E, H and T, Natural dyes, Spermatozoa, Synthetic dyes, Eco friendly dyes.

Introduction

Semen analysis is a vital laboratory test employed in the assessment of infertility. It involves the evaluation of various semen parameters, including sperm count, viability, liquefaction time, motility, and morphology. (1) Of these parameters, the assessment of spermatozoa morphology holds significant importance in fertility evaluation. Similarly, morphological assessment plays a crucial role in various fertility procedures such as intrauterine insemination and in vitro fertilisation. (2, 3) Spermatozoa morphology analysis examines individual components of the sperm, including the head, neck, body, and tail. The use of staining techniques aids in the identification and assessment of sperm morphology, with popular options including Pap staining, Leishman, Eosin-Nigrosin, Haematoxylin and Eosin (H and E), and others. (4, 5, 6)

Eosin, a synthetic cytoplasmic dye widely employed in medical laboratories for diagnostic tests, offers reliable performance. However, it is considered environmentally unfriendly and non-biodegradable, posing serious health hazards to humans and animals. (7) Consequently, there is a need for safe and eco-friendly alternatives to replace Eosin. While medical laboratories use natural dyes like Haematoxylin and saffron, their availability is limited compared to synthetic options. (8) Therefore, this study introduces a new natural alternative, Turmeric, for Eosin in the evaluation of human spermatozoa morphology. Sperm morphology is assessed using H and E staining, as well as Haematoxylin and Turmeric (H and T) staining. The staining quality of H and T is compared to the conventional H and E technique in terms of various morphological parameters.

*** Corresponding Author:**
Rubina M P
Research Scholar.
Allied Health Sciences, Srinivas University,
Mukka, 574146.
Mangalore, Karnataka
Email Id: roobyrazackmp@gmil.com

Materials and methods

The experiments are carried out within the confines of an andrology laboratory located in a fertility clinic. Prior to conducting the experiments, official authorisation is obtained from the institutional ethical committee.

Preparation of Turmeric Extract

To begin, Turmeric pieces are subjected to a drying process and subsequently pulverised using a blender. Following this, a 70% alcohol solution is

meticulously prepared and saturated with potassium alum as a mordant. The ultimate composition of the turmeric extract entails dissolving 15 grams of turmeric powder in 100 ml of the aforementioned alcohol solution, resulting in a concentration of 15 grams per 100 ml. (9,10).

Eosin extract preparation

The preparation of Eosin extract involves mixing 1gm of commercially available Eosin powder with 100 ml of alcohol to obtain a 1gm % solution of alcoholic Eosin. (11).

Preparation of Harris Haematoxylin stain

2.5 gm of Haematoxylin is dissolved in 50 ml of alcohol, while 50 gm of alum is dissolved in 500 ml of hot water. These two solutions are combined and heated to their boiling point. Subsequently, 1.5 gm of mercuric oxide is added, and the mixture is rapidly cooled. To enhance the nuclear staining property of the mixture, an additional 20 ml of glacial acetic acid is incorporated. (11).

Staining procedure

H and E staining

The prepared smears are sequentially hydrated in descending concentrations of alcohol (95%, 80%, and 50%) and subsequently in water. Nuclear staining was performed using Harris Haematoxylin solution for a duration of 1 minute. The stained smears were rinsed with water, followed by acid differentiation using 1% acid alcohol. Subsequently, bluing was carried out by immersing the smears in running tap water, and dehydration was achieved using 95% alcohol. The smears are counterstained with Eosin for 45 seconds and dehydrated in ascending concentrations of alcohol. Finally, the stained smears are cleared using Xylene and mounted in DPX (Dibutylphthalate Polystyrene Xylene). (11, 12)

H and T staining

H and T staining is performed by the same method with 5 minutes in Turmeric extract instead of 45 seconds in Eosin in the above-mentioned H and E staining. (13)

Semen samples are collected from a fertility clinic situated within a tertiary care hospital. A total of 200 smears are prepared, with 100 smears designated for H and T staining and the remaining 100 smears for conventional H and E staining. These smears are independently examined by two experienced Lab Technologists. Sperm morphology is assessed using five parameters, namely smear background, head, neck, body and tail. The quality of each staining technique is assessed and scored according to the following criteria.

Pale/Poorly stained	0
Not clear	1
Clear	2
Very clear	3

The results are summed up and statistical significance is studied using the chi-square test.

Results

Semen samples are obtained from the Andrology laboratory of a fertility clinic within a tertiary care hospital. Two sets of 100 slides are prepared from the samples, one set for conventional H and E staining and the other for H and T staining. In the H and E staining process, Eosin is used to stain the spermatozoa pink, while Haematoxylin stains the nucleus blue. On the other hand, in the H and T staining process, Turmeric imparts a yellow color to the spermatozoa, while the nucleus is stained blue with Haematoxylin. Each slide is meticulously examined for five parameters, including smear background, head, neck, body, and tail. The examination is conducted by two experienced laboratory technologists who possess over a decade of expertise in semen analysis. Each parameter is assigned a score ranging from 0 to 3 based on the quality of staining observed.

Figure 1: H and E staining (100 x)

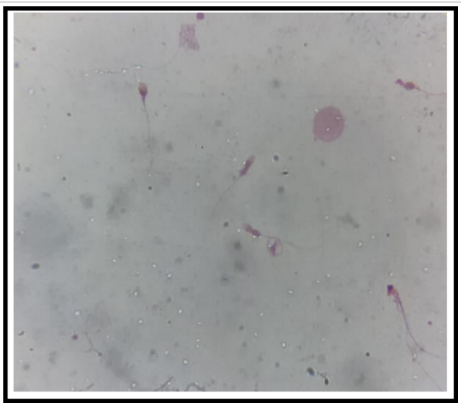


Figure 2: H and T staining (100 x)

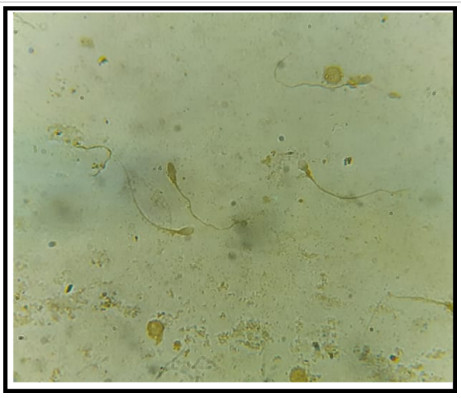
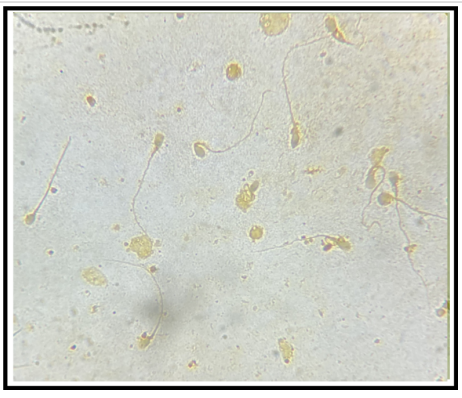


Figure 3: H and T staining result (100 X)



Discussion

Based on their origin, dyes can be classified into two types: synthetic and natural. Synthetic dyes are readily available and demonstrate higher efficiency in staining. However, they possess toxicity and exhibit lower eco-friendliness, potentially causing health

Table 1: Comparison of background scores (%) in H and E and H and T

Background score	H and E (%)	H and T (%)
0	-	-
1	-	3%
2	-	95%
3	100%	2%

Table 2: Comparison of head scores (%) in H and E and H and T

Head score	H and E (%)	H and T (%)
0	-	-
1	-	-
2	-	29%
3	100%	71%

Table 3: Comparison of neck scores (%) in H and E and H and T

Neck score	H and E (%)	H and T (%)
0	-	-
1	-	-
2	-	88%
3	100%	12%

Table 4: Comparison of body scores (%) in H and E and H and T

Body 5score	H and E (%)	H and T (%)
0	-	-
1	-	-
2		32%
3	100%	68%

Table 5. Comparison of tail scores (%) in H and E and H and T

Tail score	H and E (%)	H and T (%)
0	-	-
1	-	-
2	1%	4%
3	99%	96%

hazards for laboratory professionals and environmental issues. Conversely, natural dyes are naturally occurring, biodegradable, and pose no risks when utilized. Consequently, numerous researchers are actively seeking to substitute synthetic dyes with organic or natural alternatives. Within the realm of medical laboratories, dyes are extensively employed in various staining procedures, which contribute significantly to the diagnostic process. Regrettably, majority of staining techniques rely on synthetic dyes rather than exploring natural alternatives. Notably, Eosin stands as one of the primary synthetic dyes employed in diverse staining techniques. In this study, we propose the adoption of a natural alternative Turmeric, to replace Eosin in H and E staining for the assessment of human spermatozoa morphology.

The examination of human spermatozoa morphology plays a crucial role in the assessment of male fertility. It enables the evaluation of sperm quality, thereby aiding in the prediction of fertility in sperm donors. Such evaluations are instrumental in improving reproductive efficiency, whether in natural breeding conditions or assisted reproduction programs. Currently, several staining techniques are available to examine sperm morphology, such as Leishman staining, Pap staining, Giemsa staining, H and E staining, Eosin-Nigrosin, and others. Most of these techniques utilize Eosin as the primary dye. Among these staining methods, H and E staining stands out as a popular approach for evaluating spermatozoa morphology. H and E staining employs natural Haematoxylin as a nuclear stain and synthetic Eosin as a cytoplasmic stain. Despite Eosin's wide acceptance and efficacy as a cytoplasmic dye, its use may pose health hazards and be less environmentally friendly. Therefore, this study aims to explore the replacement of Eosin with Turmeric in H and E staining for the evaluation of human spermatozoa, offering a potential alternative that may be safer and more eco-friendly.

In this experiment, smears derived from semen samples were subjected to staining using H and E as well as H and T methods. The smear stained with H and E exhibited a blue-colored nucleus alongside pink-colored spermatozoa. Conversely, in the H and T staining, yellow-colored spermatozoa were observed, while the nucleus retained its blue hue. Microscopic evaluation of the smear background and the four compartments of sperm, namely head, neck, body, and tail, was conducted during this experiment. The quality of staining was assessed and scored on a scale of 0 to 3 based on the aforementioned criteria (refer materials and method).

In the context of H and E staining, spermatozoa are clearly visible and efficiently stained. 100% of smears scored 3 for background, head, neck, and body. Whereas in tail, 99% of smears scored 3 and the remaining scored 2 (Table 1-5). In H and T staining, even though the background of the smear has scored 2 as compared with H and E (score 3), the spermatozoa is distinctly observable (Table 1).

Table 2 presents a comparison of sperm head quality between H and E staining and H and T staining. The sperm head is distinctly observable in both staining techniques, with H and T staining revealing a yellow-colored cytoplasm exhibiting clear visible borders (see fig no 1-3). This feature aids in the assessment of morphological abnormalities in the sperm head. In H and T, the blue colored nucleus is slightly masked with Turmeric, resulting in a scoring of 3 for 71% of the H and T smears when evaluating the staining quality of the head, while the remaining smears are scored as 2.

The color of the neck and body of the spermatozoa in H and E and H and T staining is depicted as pink and yellow, respectively. Staining quality of the neck and body is presented in Table 3 and Table 4, respectively. According to the results, 88% of the neck scored 2 (Table 3), while 68% of the body scored 3 (Table 4). In comparison, the tail exhibits a higher intensity of staining with turmeric compared to the neck and body. This is shown in Table 5, where 96% of the smears stained with turmeric scored 3, while the remaining samples scored 2.

Multiple research studies have been conducted to explore the application of natural dyes sourced from

various origins for staining. In a study conducted by Sirinart Chomean et al., natural dye extracts derived from black rice (*Oryza sativa*), butterfly pea (*Clitoria ternatea*), fresh roselle (*Hibiscus sabdariffa*), and mulberry (*Morus alba*) were investigated for their potential to stain human spermatozoa. The authors assessed these extracts as a possible alternative to Haematoxylin. Among the tested extracts, black rice demonstrated promising staining properties and could serve as a substitute dye for evaluating human spermatozoa morphology. The researchers propose that the utilization of black rice extracts could reduce the expenses associated with purchasing synthetic dyes and mitigate their adverse effects on both human health and the environment. (14)

Rosemary B et al. investigated the use of *Hibiscus sabdariffa* as an alternative to Eosin for assessing sperm morphology. The findings of the experiment indicate that *Hibiscus sabdariffa* can be considered a readily available histological stain suitable for evaluating sperm morphology. (15)

Mohammadreza Ebrahimi et al. employed aqueous extract dyes derived from Black Mulberry, Henna, and Safflower for examining sperm morphology. The staining quality of these extracts was compared to that of the Eosin-nigrosin staining technique. The study demonstrates that Safflower and Black Mulberry dyes effectively stain the spermatozoa, and with further modifications, the staining quality can be enhanced. However, it should be noted that these dyes are not suitable for viability testing. (16)

Various laboratories have undertaken similar studies to develop natural alternatives to Eosin, examining different samples. (7-26) These studies explore the use of Turmeric, Ginger, Hibiscus, Cola nut, Santan flower, and others as potential substitutes for Eosin. In one such experiment, Sudhakaran et al. investigated the application of Ginger (*Zingiber officinale roscoe*) extract as a cytoplasmic dye in tissue samples.[17] The findings suggest that Ginger exhibits promise as a natural alternative to Eosin, boasting a longer shelf life.

Rubina et al. conducted an experimental study on Turmeric for staining tissue samples in histopathology. (13) The authors evaluated the staining quality of Turmeric and compared it to Eosin in H and E staining. Despite Eosin's widespread acceptance and superior contrast, Turmeric stains the cytoplasm in a comparable manner. Based on cost-effectiveness, eco-friendliness, and availability, the authors concluded that Turmeric can serve as a viable substitute for Eosin.

Conclusion

Eosin is widely recognised as a synthetic dye that is commonly used in conjunction with various dyes. Haematoxylin and Eosin (H and E) staining is a prominent technique employed in medical laboratories. The utilisation of natural dyes in diagnostic laboratories holds significant value for both the economy and environmental sustainability. In this particular experiment, we assessed the staining efficacy of Turmeric extract as a substitute for Eosin when combined with Haematoxylin for spermatozoa morphology. The experimental findings demonstrate that the employment of Haematoxylin and Turmeric (H and T) stain yields effective results. Turmeric imparts a yellow color to spermatozoa as opposed to the pink color achieved with Eosin. Although Eosin delivers superior staining in terms of contrast and clarity, Turmeric produces a comparable pattern with distinct visual compartments for spermatozoa. Considering its natural origin, Turmeric offers advantages such as cost-effectiveness, environmental friendliness, and other benefits over Eosin. Therefore, this study concludes that Turmeric can serve as an efficient alternative to Eosin in H and E staining for sperm morphology.

Reference

1. Keel B.A. How reliable are results from the semen analysis. Fertility and Sterility. July, 2004; 82(1); 41–4
2. Reimundo P, Gutiérrez Romero J.M, Rodríguez Pérez T, Veiga E. Single-embryo transfer: a key strategy to reduce the risk for multiple pregnancy in assisted human reproduction. Advances in Laboratory Medicine. April, 20212; 2(2); 179-88.
3. Kamieniczna M, Stachowska E, Augustynowicz A, Woźniak T, Kurpisz M.K. Human live spermatozoa morphology assessment using digital holographic microscopy. Scientific Reports. March, 2022; 12(1); 4846.
4. Meschede D, Keck C, Zander M, Cooper T.G, Yeung C.H, Nieschlag E. Influence of three different preparation techniques on the results of human sperm morphology analysis. International Journal of Andrology. December, 1993; 16(6); 362–9.
5. Xu Y-H, Lu J-C, Tang S-S. Effects of six kinds of sperm staining methods on human sperm size and evaluation of their staining effects. J Clin Lab Anal. December, 2022; 36(12); e24794.
6. Aksoy E, Aktan T.M, Duman S, Cuce G. Assessment of spermatozoa morphology under light microscopy with different histologic stains and comparison of morphometric measurements. International Journal of Morphology. December 2012; 30(4);1544–50.
7. Bordoloi B, Jaiswal R, Siddiqui S, Tandon A. Health hazards of special stains. Saudi Journal of Pathology and Microbiology. January, 2017; 2(5); 175–8.
8. Kumar S, Singh N, Singh A, Singh N, Sinha R. Use of Curcuma longa L. extract to stain various tissue samples for histological studies. AYU (An International Quarterly Journal of Research in Ayurveda). October, 2014; 35(4); 447-51.
9. Abraham M, Nambiar S, Charagannavar V, Augustine D, Sowmya S, Babu A, et al. Comparison of staining efficacy between turmeric and eosin: A histological study. Journal of clinical and diagnostic research. November, 2017; 11(11); ZC05-ZC08.

10. Avwioro O.G, Onwuka S.K, Moody J.O, Agbedahunsi J.M, Oduola T, Ekpo O.E, et al. Curcuma longa extract as a histological dye for collagen fibres and red blood cells. Journal of Anatomy. April, 2007; 210(5); 600–3.
11. Culling C F A, Allison RT, Barr W T, Cellular pathology techniques,4th Ed. Elsevier; 1985. 111-120.
12. Koss L.G, Melamed M.R. Koss' diagnostic cytology and its histopathologic bases. 5th Ed. Pennsylvania; Lippincott Williams & Wilkins. 2006. 1570-75.
13. Rubina M.P, Krishnan A.M, Riyas B.K, Mohammed S.T, Soumya V. Assessment of Staining Quality of Curcumin as a Substitute for Eosin in haematoxylin and Eosin Staining in Histopathology. J Res Med Dent Sci. Aug 2020; 8(5); 146-50.
14. Chomean S, Nantabut M, Kongtia W, Saenguthai K, Kaset C. Evaluation of natural dyes for human spermatozoa morphology assessment. Acta Histochemica. FEbruary, 2019; 121(2); 227–33.
15. Bassey R.B, Osinubi A.A, Oremosu A.A. Staining effect of *hibiscus sabdariffa* extract on sperm cell morphology of Sprague–Dawley rats. Journal of Histotechnology. September 2012; 35(3); 110–3.
16. Ebrahimi M, Parham A. Using herbal dyes as an alternative staining method for sperm evaluation. Veterinary Medicine and Science. August 2020; 6(3); 441–6.
17. Hallikeri K, Sudhakaran A, Babu B. Natural stains Zingiber Officinale roscoe (ginger) and Curcuma longa L. (turmeric) – a substitute to Eosin. AYU (An international quarterly journal of research in Ayurveda). Oct-Dec, 2018; 39(4); 220.
18. Agbede M, Benard S, Afolabi O, *et al*. The use of hibiscus sabdariffa extract as nuclear stain for skin morphology and connective tissue with eosin counterstain. Sokoto J Med Lab Sci. December, 2017; 2(4) :28–32.
19. Sridhara S.U, Raju S, Gopalkrishna A.H, Haragannavar V.C, Latha D, Mirshad R. Hibiscus: A different hue in histopathology. Journal of Medicine, Radiology, Pathology and Surgery. Jan-Feb, 2016; 2(1) ; 9–11.
20. Chinyere C.G, Etoforinini C.C. Alternative staining of kidney tissue sections with extracts of turmeric (*Curcuma longa*) and Zobo (*Hibiscus sabdariffa*). J. Histotechnol. 2017;1-9.
21. Bose S, Sharma M, Karthikeyan R, Kumar M, Chawla G, Tyagi V. Histopathological correlative assessment of cytoplasmic staining by aqueous extracts of hibiscus and turmeric with eosin on 70 tissue samples. International journal of health sciences. April, 2022; 6(s3); 3915–25.
22. Ibnouf A.A, AbdulRaheem E, Seed Ahmed M, Dahab D. Assessment of staining quality of Roselle (Hibiscus Sabdariffa) on formalin fixed paraffin-embedded renal tissue sections. International Journal of Current Research and Review. November, 2014; 6(21); 26
23. KUŞÇULU N. Dual staining effect of extracts Roselle (*Hibiscus sabdariffa* L) and red cabbage (*Brassica oleracea*) on Wistar rat tissues. February 2021; 1; 1-11.
24. Cruz PEC, De Vera AP, Villa ADM. The efficacy of santan flower (*Ixora occinea linn*.) as an alternative stain to eosin y in wright-giemsa stain. Lyceum of the Philippines–St Cabrini Journal of Allied Medicine. 2018; 3; 1–1.
25. Shehu SA, Sonfada ML, Danmaigoro A, Umar AA, Hena SA, Wiam IM. Kola nut (*Cola acuminata*) extract as a substitute to histological tissue stain eosin. Journal of Veterinary Advances .2012 Aug 28; 1(2); 33–7.
26. Meenakshi, et. al. A comparative cytological study of Eosin & eco-friendly cytoplasmic stains -A Pilot Study.IOSR Journal of Dental and Medical Sciences. April, 2021; 20(04); 44-49.

*****