Submitted: 03 February, 2024 Accepted: 13 March, 2024 Published: 30 October, 2024 DOI:10.22514/jomh.2024.167

ORIGINAL RESEARCH

JOMH Journal of Men's Health

Assessment of the sperm DNA Fragmentation using SCSA by fluorescence microscopy and flow cytometry in males from an andrology clinic

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Abstract

The assessment of male fertility has traditionally depended on the evaluation of conventional semen parameters. Recent advances have identified sperm DNA fragmentation as a valuable biomarker for the assessment of male infertility. This study recruited 121 men from an andrology clinic to evaluate the diagnostic efficiency of the sperm DNA fragmentation index (DFI), utilizing the sperm chromatin structure assay (SCSA) through both flow cytometry and fluorescence microscopy. The study also explored the relationship between sperm DFI and standard semen parameters such as concentration, motility and morphology. The results showed that men with abnormal semen parameters were found to have significantly reduced sperm progressive motility ($p < 0.001$), total motility ($p < 0.001$) and normal morphology ($p < 0.001$), as well as higher sperm DFI, as determined by both fluorescence microscopy and flow cytometry (both $p < 0.001$), compared to those with normal semen parameters. A negative correlation was observed between sperm progressive motility, total motility, sperm normal morphology and sperm DFI, regardless of whether the DFI evaluation was conducted using fluorescence microscopy or flow cytometry (all $p < 0.001$). In conclusion, the application of the SCSA assay via both fluorescence microscopy and flow cytometry reveals that sperm DFI is closely associated with seminal parameters, reinforcing its utility in the clinical evaluation of male fertility.

Keywords

Sperm DNA fragmentation; DFI; SCSA; Fluorescence microscopy; Flow cytometry

1. Introduction

The worldwide incidence of infertility in reproductive-aged couples has been steadily increasing due to factors such as adverse lifestyle choices and environmental pollution [1–3]. One of the most common causes of male infertility is poor semen quality, which could be due to reduced sperm motility (efficiency of sperm movement), diminished sperm concentration (quantity of sperm per unit volume of semen) and an increased proportion of spermatozoa with abnormal morphology (irregular shape and structure) [4]. Therefore, the assessment of male fertility predominantly depends on the analysis of these conventional semen parameters.

Recently, the sperm DNA fragmentation index (DFI) has emerged as a valuable biomarker for the diagnosis of male infertility, garnering attention from clinicians [5]. Elevated levels of DFI have been linked to adverse outcomes in assisted reproductive technology (ART), recurrent pregnancy loss and cases of unexplained male infertility [6]. Furthermore, the inclusion of DFI in the broader semen analysis is recommended by the World Health Organization (WHO) in its laboratory manual for the examination and processing of human semen (sixth edition) [7].

Sperm DFI assessment can be conducted through both direct methods, such as the terminal deoxynucleotidyl transferase 2-deoxyuridine 5-triphosphate (dUTP) nick end labeling (TUNEL) and comet assays, and indirect methods, including the sperm chromatin structure assay (SCSA) and sperm chromatin dispersion (SCD) assay [8]. Among these, SCSA stands out as the simplest and most widely used method for DFI determination [9]. It is characterized by high repeatability and low variability when implemented through flow cytometry [10]. Currently, SCSA measurements can be performed using either flow cytometry or fluorescence microscopy. Flow cytometry offers a fast, automated method capable of analyzing a significant number of spermatozoa swiftly, making it invaluable in clinical settings for prompt diagnosis and intervention. Conversely, fluorescence microscopy, while assessing fewer spermatozoa, provides high specificity and the ability to closely examine individual sperm cells. This method enables the detailed observation of subtle chromatin structural anomalies with remarkable precision. However, the comparative diagnostic effectiveness of these two methodologies for evaluating sperm DFI remains to be fully elucidated.

Therefore, the aim of this present study was to assess the diagnostic efficiency of sperm DFI using SCSA by both flow cytometry and fluorescence microscopy.

2. Methods

2.1 Patients

In the current study, data were collected from 121 men between March 2023 and July 2023 at a single reproductive medicine center in the First Affiliated Hospital of USTC (Hefei, China). All participants underwent a fertility evaluation through semen analysis. Men diagnosed with azoospermia were excluded from the study.

2.2 Standard semen analysis

Semen samples were obtained through masturbation after 2–7 days of sexual abstinence, and the semen volume was measured by the weight of the sample in a container. After liquefaction at 37 °C for 30 minutes, key semen parameters, including sperm concentration, motility and morphology, were evaluated. The sperm concentration and the percentages of progressive and total motility were determined using computer-assisted sperm analysis (CASA) technology (SAS-II, SAS Medical, Beijing, China). To assess the percentage of sperm with normal morphology, approximately 200 spermatozoa per sample were examined following staining with Diff-Quick (20230120, Ankebio, Hefei, China). The concentration of seminal leukocytes was identified through a peroxidase test (20221205, Ankebio, Hefei, China), while antisperm antibodies (AsAs) were evaluated using the indirect mixed antiglobulin reaction (MAR) technique (20230109, Ankebio, Hefei, China).

2.3 Measurement of sperm DFI

The sperm DFI (%DFI) was quantified using (Fig. 1). Sperm samples were initially diluted in phosphate-buffered saline (PBS) to achieve a concentration of 2×10^6/mL. A detergent solution consisting of 0.1% Triton X-100, 0.15 mol/L NaCl and 0.08 mol/L HCl was then added, followed by a 30-second incubation period. Acridine orange dye at a concentration of 6 μg/mL was subsequently introduced to the mixture for a 3-minute staining. For the flow cytometry analysis, the samples were examined using a flow cytometer (Sparrow, Celula, Chengdu, China), where a total of 5000 spermatozoa per sample were assessed. In the fluorescence microscopy analysis, 10 μL of the stained spermatozoa were smeared onto slides, with at least 200 spermatozoa per sample evaluated under a microscope (ECLIPSE Si, Nikon, Tokyo, Japan). The determination of DFI involved calculating the ratio of red to total (red and green) fluorescence detected by both flow cytometry and fluorescence microscopy.

2.4 Statistical analysis

Categorical variables are reported as frequencies and continuous variables as either means ± standard deviation (SD) or medians with interquartile ranges (IQR). The Mann-Whitney U test was used to compare two groups with non-parametric data. Adhering to WHO guidelines, the lower reference limits for semen parameters were established as follows: sperm concentration $<15 \times 10^6$/mL, progressive sperm motility <32%, and normal morphology <4%. To normalize the distribution and ensure homoscedasticity of residuals, sperm concentration data underwent a natural logarithm transformation. The correlation between DFI and standard semen parameters was analyzed using Spearman's correlation test. All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant. Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc, San Diego, CA, USA).

3. Results

Table 1 presents the clinical characteristics and semen parameters of the 121 men investigated in this study. The average age of the participants was 31.3 ± 4.9 years. For the basic semen parameters, the medians for semen volume and sperm concentration were 3.2 mL and 69.9 $\times 10^6$/mL, respectively. Furthermore, the medians for progressive motility, total motility and normal morphology were reported as 36.4%, 42.3% and 7.0%, respectively. Additionally, the median values for the

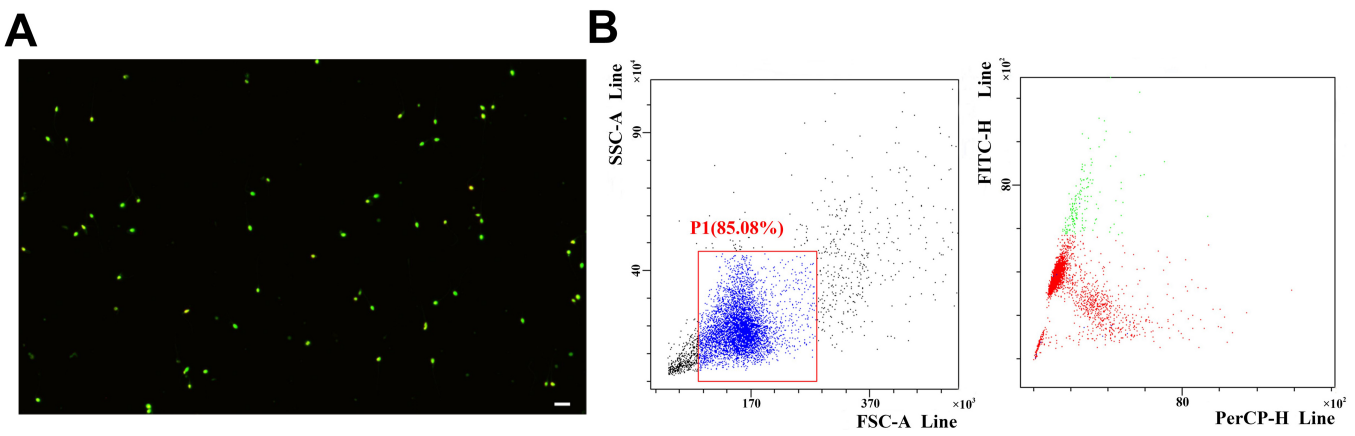

FIGURE 1. Measurement of sperm DFI. (A) Sperm DFI was detected by fluorescence microscopy. (B) Sperm DFI was detected by flow cytometry. Green indicates normal sperm DFI, and red/orange indicates high sperm DFI. Scale bar: 10 μm. SSC: side scatter; FSC: forward scatter; FITC: fluorescein isothiocyanate; PerCP: peridinin-chlorophyll-protein complex.

DFI assessed by fluorescence microscopy and flow cytometry were 16.0% and 16.3%, respectively.

TABLE 1. Clinical characteristics and semen parameters of the whole cohort.

Clinical characteristics	Total (n = 121)
Age (yr), mean ± SD	31.3 ± 4.9
Semen parameters (median (IQR))	
Semen volume (mL)	3.2 (2.3, 4.1)
Sperm concentration (×10^6/mL)	69.9 (36.7, 115.0)
Progressive motility (%)	36.4 (28.1, 49.5)
Total motility (%)	42.3 (32.7, 55.0)
Normal morphology (%)	7.0 (5.0, 9.0)
Leukocytes (×10^6/mL)	0.06 (0.04, 0.37) (n = 66)
AsAs (%)	0 (0, 8.0) (n = 27)
DFI (fluorescence microscopy, %)	16.0 (12.1, 22.9)
DFI (flow cytometry, %)	16.3 (9.1, 25.1)

SD: standard deviation; IQR: interquartile range; AsAs: antisperm antibodies; DFI: DNA fragmentation index.

Pearson analysis revealed a high correlation ($r^2 = 0.86$; $p < 0.001$) between the two DFI tests (Fig. 2).

All men were then divided into two subgroups based on their semen quality: those with normal semen parameters and those with abnormal semen parameters, according to WHO criteria. Compared to individuals with normal semen parameters, those with abnormal parameters demonstrated significantly lower progressive motility ($p < 0.001$), total motility ($p < 0.001$), and normal morphology ($p < 0.001$), as well as higher sperm DFI when assessed by fluorescence microscopy (FM) and flow cytometry (FC) (both $p < 0.001$) (Table 2). In participants with normal semen parameters, sperm DFI values measured by FM were higher than those measured by FC; conversely, in participants with abnormal semen parameters, the trend was reversed.

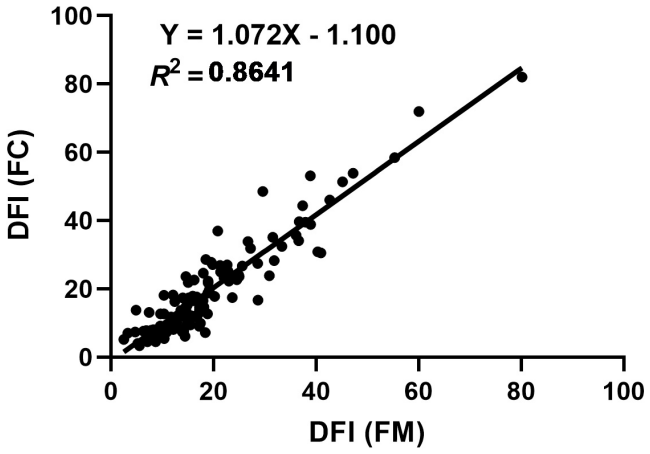

FIGURE 2. Correlation between sperm DFI as detected by SCSA via fluorescence microscopy (FM) and flow cytometry (FC). Data were analyzed using Spearman's correlation coefficient analysis. DFI: DNA fragmentation index; SCSA: sperm chromatin structure assay.

The study further explored the relationships between sperm DFI and standard semen parameters (Fig. 3). A significant negative correlation was identified between sperm progressive motility, total motility, normal morphology and sperm DFI, irrespective of whether DFI evaluation was performed using FM or FC (all $p < 0.001$).

4. Discussion

Sperm DNA fragmentation, defined as the occurrence of double or single-strand breaks in the genome, plays an important role in early embryo development [11]. In our present study, semen samples from a cohort of 121 patients were collected and assessed according to WHO criteria for sperm concentration, motility and morphology. Additionally, sperm DNA integrity was evaluated using SCSA through both fluorescence microscopy and flow cytometry. We observed a positive correlation between the assessments of DFI by fluorescence microscopy and flow cytometry. Moreover, this study demon-

TABLE 2. Standard semen parameters and DFI between the participants with normal and abnormal semen parameters.

Semen parameters	Subgroups		
	Normal (n = 61)	Abnormal (n = 60)	*p*
Semen volume (mL)	3.2 (2.7, 4.6)	3.1 (2.1, 3.9)	0.104
Sperm concentration (×10^6/mL)	70.6 (43.9, 120.8)	65.6 (30.6, 101.8)	0.186
Progressive motility (%)	47.2 (38.8, 59.0)	28.1 (18.0, 33.7)	<0.001
Total motility (%)	52.1 (43.0, 65.3)	32.7 (21.9, 38.6)	<0.001
Normal morphology (%)	8.0 (6.5, 10.0)	5.0 (4.0, 7.0)	<0.001
DFI (fluorescence microscopy, %)	14.2 (8.8, 18.0)	18.9 (14.5, 29.3)	<0.001
DFI (flow cytometry, %)	12.7 (7.6, 17.2)	22.1 (12.4, 34.0)	<0.001

Sperm concentration data were transformed using natural logarithms to obtain normally distributed residuals and homoscedasticity. DFI: DNA fragmentation index.

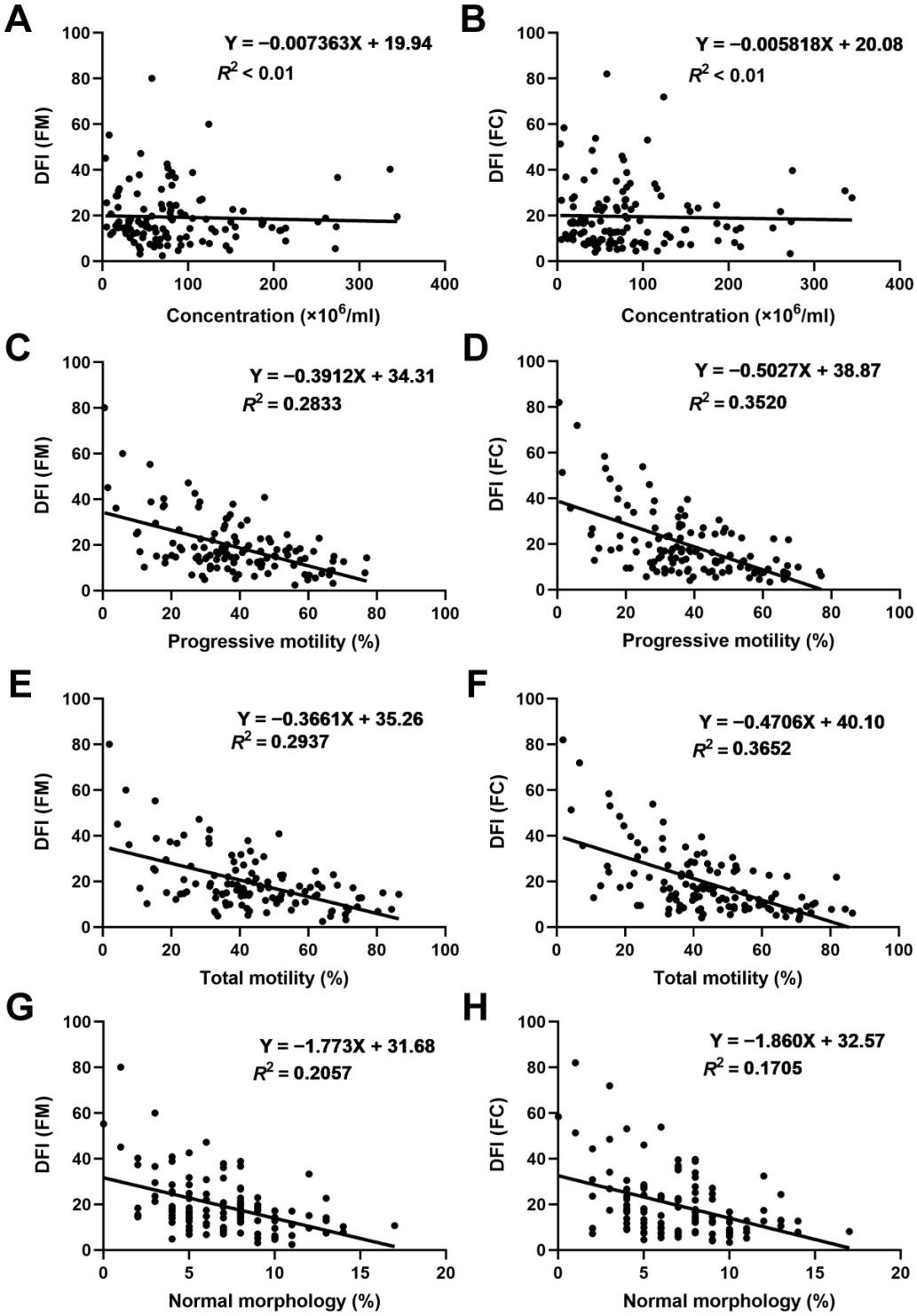

FIGURE 3. Correlation between standard semen parameters and sperm DFI detected by SCSA via fluorescence microscopy (FM) and flow cytometry (FC). (A) Spearman's correlation coefficient analysis showing correlation between sperm concentration and sperm DFI detected by SCSA via FM. (B) Spearman's correlation coefficient analysis showing correlation between sperm concentration and sperm DFI detected by SCSA via FC. (C) Spearman's correlation coefficient analysis showing correlation between sperm progressive motility and sperm DFI detected by SCSA via FM. (D) Spearman's correlation coefficient analysis showing correlation between sperm progressive motility and sperm DFI detected by SCSA via FC. (E) Spearman's correlation coefficient analysis showing correlation between sperm total motility and sperm DFI detected by SCSA via FM. (F) Spearman's correlation coefficient analysis showing correlation between sperm progressive motility and sperm DFI detected by SCSA via FC. (G) Spearman's correlation coefficient analysis showing correlation between sperm normal morphology and sperm DFI detected by SCSA via FM. (H) Spearman's correlation coefficient analysis showing correlation between sperm progressive motility and sperm DFI detected by SCSA via FC. DFI: DNA fragmentation index; SCSA: sperm chromatin structure assay.

strated a negative correlation between semen quality and sperm DFI.

Several methods are available for assessing sperm DNA integrity, including SCSA, SCD, TUNEL and comet assay [12]. Among these, SCSA is frequently utilized to evaluate DNA fragmentation. In the SCSA procedure, sperm DNA is stained with acridine orange, where green fluorescence signifies double-stranded DNA and red fluorescence indicates single-stranded DNA [13]. The sperm DFI is widely used to assess sperm DNA damage. Notably, a DFI exceeding 30% is linked to fertilization failure, poor-quality blastocyst development, and recurrent pregnancy loss [14–16]. SCSA detection can be performed using two techniques: flow cytometry and fluorescence microscopy.

Flow cytometry offers numerous advantages for evaluating sperm DNA integrity. This automated method allows for the rapid analysis of a large volume of spermatozoa, making it particularly useful in clinical environments where quick assessments are vital for timely diagnosis and treatment. On the other hand, fluorescence microscopy provides distinct benefits, including high specificity in analyzing smaller sperm samples despite the fact that it may lack the high-throughput analysis of flow cytometry. This technique excels in the detailed visualization and analysis of individual sperm cells, facilitating the precise identification of subtle DNA integrity abnormalities. In this study, sperm DFI was assessed using both fluorescence microscopy and flow cytometry through SCSA. High sperm DFI was found to negatively correlate with sperm motility and morphology, corroborating previous research that supports the reliability of both methods in evaluating sperm DNA integrity [17].

Interestingly, sperm with normal motility and morphology may still exhibit high DNA fragmentation [18]. Although single-stranded DNA damage may be repaired by the oocyte or embryo, extensive double-stranded DNA damage is often irreversible, leading potentially to abnormal embryo and fetal development [19]. The detection of poor sperm DNA integrity in individuals who meet the WHO criteria for normal semen quality suggests a significant role in male infertility. Incorporating the sperm DFI with standard semen parameters provides a more objective insight into semen quality and potential fertility outcomes.

Fragmentation of DNA primarily results from three mechanisms: (1) Abortive apoptosis during meiosis; (2) Defective chromatin packaging during spermiogenesis; (3) Oxidative stress encountered during passage through the epididymis [20, 21]. The heightened vulnerability of post-testicular sperm to oxidative stress, a leading cause of sperm DNA damage, leads to increased DFI levels in the caudal epididymis and ejaculate compared to testicular sperm [22]. Given that human spermatogenesis in the testis spans 72 days and sperm maturation in the epididymis takes an additional 12 days, antioxidant therapy *in vivo* for a minimum duration of 3 months is recommended to mitigate oxidative stress-induced sperm DNA fragmentation [23, 24].

This study has several limitations. First, it investigates only a relatively small sample size. Second, semen samples with sperm concentrations lower than 1 million/mL were excluded from measurement. Third, the quantity of spermatozoa evaluated by flow cytometry surpassed those assessed by fluorescence microscopy.

5. Conclusions

In conclusion, our study demonstrates that SCSA conducted via both fluorescence microscopy and flow cytometry produces consistent results and that sperm DFI is negatively correlated with sperm motility and normal morphology. Further research involving larger-scale studies is required to corroborate our findings.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

JYL and SB—designed the study, verified the underlying data. JYL, BXL and SB—contributed to the data acquisition. JYL, YZ, BXL and SB—analyzed the data. SB—wrote the original manuscript. All authors contributed to the review of the final article and accept responsibility for the decision to submit for publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by The First Affiliated Hospital of University of Science and Technology of China Ethical Committee (2023-KY-415). Participants were informed and signed a consent form.

ACKNOWLEDGMENT

We thank Xia Wu, Jingru Xu and Yanyan Shang (all with The First Affiliated Hospital of University of Science and Technology of China) for their valuable contributions to the data collection.

FUNDING

This study was supported by the National Natural Science Foundation of China (81901543) and the Science and Technology Project of Jingdezhen (2022SFZC035).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Jiyan Li, Yi Zhou, Bingxin Liu, Shun Bai. Assessment of the sperm DNA Fragmentation using SCSA by fluorescence microscopy and flow cytometry in males from an andrology clinic. Journal of Men's Health. 2024; 20(10): 73-78. doi: 10.22514/jomh.2024.167.