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Original Research Article

COMPARING SPERM CONCENTRATION MEASUREMENT IN HUMAN SEMEN USING IMPROVED NEUBAUER HEMOCYTOMETER AND MAKLER COUNTING CHAMBER

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Abstract

Background: Sperm concentration measurement is one of the main parameters in routine semen analysis for diagnosing male infertility. WHO recommends the use of the Improved Neubauer Hemocytometer for the determination of sperm concentration. Despite this, many other sperm counting methods are being used in laboratories worldwide, the uses of which have been justified in various studies. Makler counting chamber is one of these methods. Hence, we aimed to study the comparison between the Improved Neubauer Hemocytometer and the Makler counting chamber for sperm concentration measurement in semen analysis. **Materials and Methods:** Sperm concentration values by manual sperm counting obtained by using the Makler chamber and the Improved Neubauer Hemocytometer for 86 semen samples were statistically compared. **Result:** The mean sperm concentrations ± S.D. obtained by the Makler counting chamber and the Improved Neubauer Hemocytometer were 67.99±68.20 million/ml and 43.13±42.44 million/ml, respectively. The difference between the mean sperm concentrations measured with the two chambers was statistically significant, with the Makler counting chamber overestimating the sperm concentration values. The percentage of difference between the mean sperm concentrations obtained by the two chambers was 57.6%. Even after the classification of the concentrations into oligozoospermia and normozoospermia, both groups showed a statistically significant difference between the two chambers. **Conclusion:** Therefore, it can be concluded that despite being a more rapid and easy to use method, the Makler counting chamber should not be used in laboratories for sperm concentration measurement for infertility diagnosis.

INTRODUCTION

Infertility has been a global issue through the ages and continues to be an important clinical problem affecting 8-12 percent of couples worldwide during their reproductive lives. About 40-50% of these cases are due to “male factor” infertility.[1,2]

Semen analysis, considered to be a diagnostic cornerstone, is a commonly employed laboratory investigation in andrology for assessing male fertility.[3,4] Its accuracy and precision are important, to be able to arrive at the required health-care decisions concerning assisted reproductive technology (ART). It evaluates several parameters including sperm count, sperm concentration, morphology of spermatozoa, percentage and quality of motility, with sperm concentration being an important one.[5-8]

Many sources of variability can be found in semen analysis and therefore, it is important to govern them. This was tried to be achieved by standardization of procedures.[9]

However, there remains a lack of standardization in clinical laboratories all over the world.[10-12] The ‘WHO laboratory manual for examination and processing of human semen’ recommends the use of Improved Neubauer Hemocytometer as a sperm counting chamber with protocols for quality control.[2] However, various other sperm counting methods are used in the andrology laboratories all around the world like DROP, Standard Count, Cell Vision, MicroCell, 2X-CEL, Makler, JCD, Burker, CellVU, Leja, Macro, GoldCyto, Geoffrey, Thoma, and Computer-Assisted Semen Analysis methods.[6,13,14] Sperm concentration can be estimated using any of these counting chambers or

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disposable slides, some having higher reproducibility and precision as compared to others, accounting to the varying loading methods and chamber depths.[15,16] Thus, there are wide differences in the sperm counts obtained by these laboratories with conflicting and incompatible results.[6] This might have an adverse impact on patient care when a sample is incorrectly reported as 'consistent with infertility' by a particular laboratory, resulting in a recommendation for artificial insemination.[17] This could potentially cause a family unnecessary physical, financial, mental, and emotional distress.

One of the commonly employed methods for sperm counting is the Makler counting chamber.[13] It is a rapid and easy to use device from which the sperm count of a preheated and undiluted sample can be obtained.[18] Several contradicting reports have been presented comparing the Improved Neubauer Hemocytometer with the Makler chamber as the routine method for determining sperm concentration.[13,16-25] The aim of this study is to compare the Makler counting chamber and the Improved Neubauer Hemocytometer for sperm concentration measurement. It will also aid in understanding the various advantages and disadvantages of both methods.

Aims and objectives:

The objective of this study was to compare the sperm concentration obtained by two methods i.e., using Improved Neubauer Hemocytometer and Makler counting chamber.

MATERIALS AND METHODS

A one-year hospital based cross-sectional study was conducted in a tertiary health care centre.

Sample size: Calculated Minimum sample size was 44 samples. 86 semen samples received in the clinical pathology laboratory of our hospital during the study interval were included in the study.

Inclusion Criteria

All semen samples received in the clinical pathology laboratory during the study period. Azoospermia semen samples detected by only one of the two methods were also included in the study.

Exclusion Criteria

Azoospermia semen samples detected by both the methods were excluded from the study.

Data collection procedure & instruments used: Prior approval of the study was taken from the institutional ethical committee (IEC Ref No.-205/2017-18). Written informed consent was obtained from participants for enrolment in the study.

Semen samples were obtained by masturbation, after 3 days of abstinence, by ejaculation into a clean, wide mouthed container.[2,17]

The specimen container was thereby placed on the bench at room temperature for about 30-45 minutes for liquefaction. The sample was then thoroughly mixed for obtaining an even distribution of sperms throughout the sample. The estimation of spermatozoa concentration was done manually using the Makler counting chamber and the improved Neubauer Hemocytometer using standard methods.[2] The means of these values were then compared, and the results obtained were statistically analysed.

Makler counting chamber (Manufacturer: Sperm 360, Aurangabad): The Makler counting chamber used in this study (Figure 1) is a modified version of the Makler chamber, named as Sperm meter, and is comprised of two parts: 1. The bottom part is a metal base that has a flat optical glass disc at the centre and two metal handles around it. 2. The top part is a cover glass in a metal ring. A grid of $1mm^2$, which is subdivided into 100 squares (10X10), each with an area of 0.1X0.1 mm, is present at the under surface of the centre of the cover glass. After the cover glass is positioned on the base, the total number of sperm heads counted in 10 consecutive squares indicates their concentration in million/mL.[26]

The lower metal base is placed on a rectangle shaped holder in the chamber we used in this study, to allow its accommodation on the microscope stage, in comparison to the original Makler chamber which has a round metal base.

After keeping the test tube, containing a part of the undiluted specimen of semen, in a hot water bath ($50\text{-}60^0C$) for five minutes, to immobilize the spermatozoa, a drop from it was placed on the chamber. The slide was immediately covered with the cover glass. The sperm heads within a strip of 10 consecutive squares counted under 200x magnification gave a number describing sperm concentration in millions/mL.[13] [Figures 2 & 3]

Improved Neubauer Hemocytometer (Manufacturer: Rohem India): [Figure 4]

The sample was diluted for immobilizing the spermatozoa by the addition of the diluent. It was prepared by adding 50 g of sodium bicarbonate and 10 mL of 35% (v/v) formalin, and 5 mL of saturated aqueous gentian violet to 1000 mL of distilled water, and a dilution of 1:20 was obtained.[2]

A drop from the diluted semen, after thorough mixing, was transferred to both the chambers of the Hemocytometer and covered with a cover glass. Then, to prevent drying out, it was placed in a humid chamber for 5 minutes. Next, the sedimented cells were counted under a light microscope under 400x magnification.[19] Spermatozoa were counted in all four corner WBC squares [Figure 5] and sperm concentration was calculated as per WHO guidelines using the formula below.[2]

$$C = \frac{\text{Sperms counted} \times \text{Dilution factor}}{(\text{Number of squares counted} \times \text{Volume of 1 square})}$$

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The average count of two sides of the chamber was calculated (provided the difference between them was less than 10%).

The value obtained is expressed as sperm concentration in millions/mL.

Statistical Analysis - was done using SPSS software. The following were calculated:

1) Mean ± Standard Deviation (SD), percentages, and diagrams.
2) Median and interquartile percentiles.
3) Comparison of the means of Improved Neubauer Hemocytometer and Makler counting chamber using Mann Whitney U test. A p-value of <0.05 was considered to be significant.
4) Coefficient of variation (CV).
5) Percentage of difference (P.D.) between the Improved Neubauer Hemocytometer and the Makler counting chamber. Using the formula (13):

P.D. =

(Mean by Makler counting chamber - Mean by Neubauer Hemocytometer)/(Mean by Neubauer Hemocytometer) ×100

$$\frac{\text{Mean by Makler counting chamber} - \text{Mean by Neubauer Hemocytometer}}{\text{Mean by Neubauer Hemocytometer}} \times 100$$

RESULTS

A total of 86 semen samples obtained from 86 individuals were studied. 92% of the population was under the age of 40 years, with the mean age being 31.53 years. [Figure 6, Table 1]

Mean sperm concentration obtained by the Makler chamber was more than that obtained by the Improved Neubauer Hemocytometer by about 24.86 million/ml. [Table 1].

A percentage difference of 57.6 % was seen between the mean sperm concentrations obtained by the Makler counting chamber and the Improved Neubauer Hemocytometer.

The p-value for mean sperm concentrations obtained by using the Improved Neubauer Hemocytometer and the Makler counting chamber was calculated as 0.0285 (< 0.05), indicating a statistically significant difference between the two. The Coefficient of variation (CV) for the Makler chamber was 100% and was more than that for the Improved Neubauer Hemocytometer, which was 98.37%. [Table 2].

The samples were further divided into two groups: oligozoospermia for sperm concentration < 15 million/ml and normozoospermia for sperm concentration > 15 million/ml. (2) The p-value for mean sperm concentration in both groups was 0.001. Therefore, the difference between the sperm concentrations obtained by using the two chambers was statistically significant for all concentrations. [Table 3]

[Table 4] shows the distribution of patients according to sperm concentration (million/ml) in the Improved Neubauer Hemocytometer & Makler chamber

[Table 5 and Table 6] show the median sperm concentrations obtained by using the Improved Neubauer Hemocytometer and the Makler counting chamber in all the samples and after classificiation into oligozoospermic samples (< 15 million/ ml) and normozoospermic samples (> 15 million/ml), respectively.

Table 1: Descriptive statistics

Variables		Minimum	Maximum	Mean	Std. Deviation
Age		20	55	31.53	6.297
Sperm concentration (million/ml)	Improved Neubauer Hemocytometer	0	176	43.13	42.438
	Makler chamber	1	340	67.99	68.204

Table 2: Comparison between Improved Neubauer Hemocytometer and Makler Chamber for all the samples

Sperm counting Chamber	Mean±SD (million/ml)	Coefficient of variation (CV)	Mann whitney U test
Improved Neubauer Hemocytometer	43.13±42.438	98.37%	U=2591.5 P=0.0285*
Makler chamber	67.99±68.204	100%	
*: Significant difference			

Table 3: Comparison between Improved Neubauer Hemocytometer and Makler chamber in Oligospermia and Normozoospermia groups

Classification of sperm concentration (million/ml)	Mean±SD		Mann Whitney U test
	Improved Neubauer Hemocytometer	Makler chamber	
Oligozoospermia (≤15)	5.52±4.40	6.38±4.99	U=1.50 P=0.001*
Normozoospermia (>15)	57.48±39.59	88.6±67.02	U=4.500 P=0.001*
*: Significant difference			

Table 4: Distribution of patients according to sperm concentration (million/ml) obtained by using Improved Neubauer hemocytometer & Makler Chamber

Classification of sperm concentration (million/ml)	Improved Neubauer Hemocytometer		Makler Chamber	
	No. of patients	Percentage	No. of patients	Percentage
≤15	27	31.4	23	26.74
>15	59	68.6	63	73.26

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Total	86	100.0	86	100.0

Table 5: Comparison between the medians of Improved neubauer hemocytometer and makler chamber for all the samples

Variables		Sperm Concentration (million/ml)	
		Improved Neubauer Hemocytometer	Makler Chamber
Median		30.00	45.00
Percentiles	25	10.00	15.00
	50	30.00	45.00
	75	58.00	102.00

Table 6: Comparison between the medians of sperm concentrations obtained by using Improved Neubauer Hemocytometer and Makler Chamber in Oligospermia and Normozoospermia groups.

		Oligospermia (< 15million / ml)		Normozoospermia (>15million/ ml)	
		Improved Neubauer Hemocytometer	Makler Chamber	Improved Neubauer Hemocytometer	Makler Chamber
Median		6.000	4.000	48.000	74.000
Percentiles	25	1.000	1.000	30.000	39.000
	50	6.000	4.000	48.000	74.000
	75	8.875	10.000	75.000	109.500

Figure 1: Makler chamber- Left -Base; Right- cover glass with metal ring

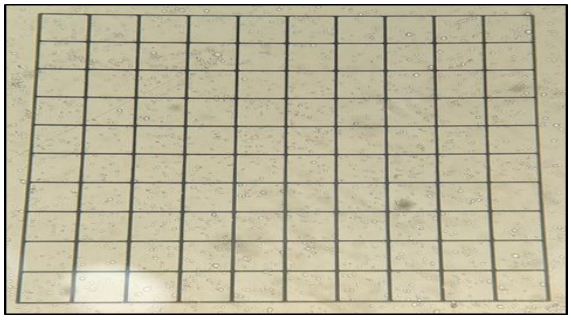

Figure 2: 100X Magnification- Microscopic view of Makler chamber showing a grid of 10X10 small squares

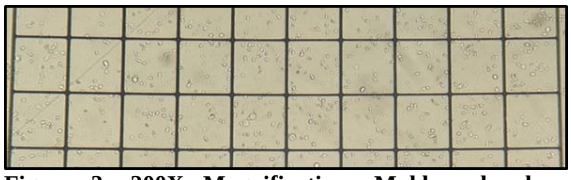

Figure 3: 200X Magnification- Makler chamber- Sperm heads to be counted in a strip of 10 consecutive squares.

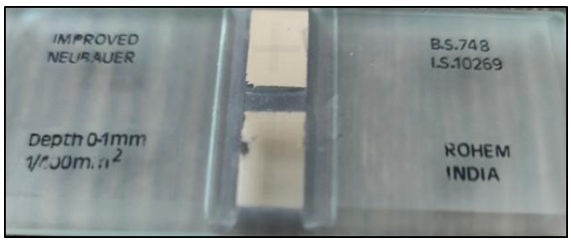

Figure 4: Improved Neubauer Hemocytometer

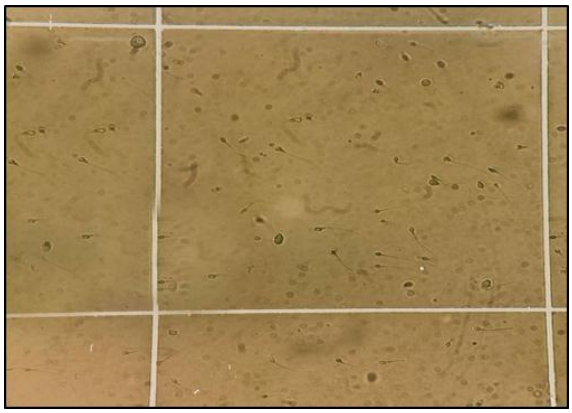

Figure 5: 400 X magnification- Spermatozoa as visualized in one small square of Improved Neubauer Hemocytometer

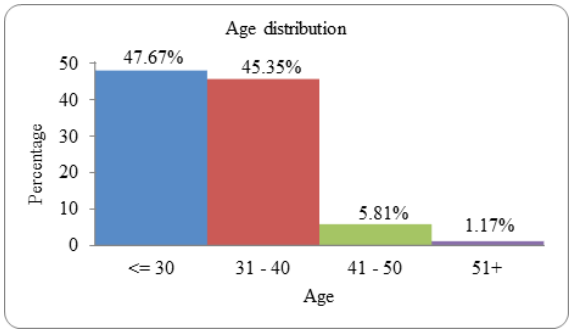

Figure 6: Distribution of patients according to Age (Years)

DISCUSSION

The present study indicates that when the mean values for the entire population are considered, the sperm concentrations obtained by the Makler chamber are significantly different from those produced by the Improved Neubauer Hemocytometer. This confirms the observations of Lu JC et al,[17] Sukcharoen N et al,[19] and Imade GE et al.[20] However, it also contradicts studies by Marchlewska K et al,[13] Atiq N et al,[18] Mahmoud AMA et al,[21] and Cardona-Maya W et al.[22]

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In our study, similar results were obtained even after the subdivision of the population into two groups- oligozoospermia and normozoospermia. This is in partial agreement with Marchlewska K et al,[13] who concluded that the concentrations produced for oligozoospermia (<20x10^6/ml) were significantly overestimated by the Makler chamber, in contrast to those produced for normozoospermia (>20x10^6/ml). Sukcharoen N et al,[19] also found that in comparison with the Improved Neubauer Hemocytometer, the Makler chamber produced significantly higher sperm concentrations of semen samples with concentrations less than 40×10^6/ml. However, in a study by Lu JC et al,[17] it was observed that the sperm concentrations by the Hemocytometer were significantly higher for ejaculates with low, medium and high concentrations.

In our study, the mean concentration estimated by the Makler counting chamber was 57.6% more than that obtained by using the Improved Neubauer Hemocytometer. We also observed that the concentrations measured by the Makler chamber were greater than those obtained by the Neubauer chamber for each semen sample analysed. Like the current study, many other studies showed that the Makler chamber produced higher sperm concentration values.[13,19-21,23]

In this study, it was found that the CV of Makler chamber was greater than that of the Improved Neubauer Hemocytometer. However, a previous study by Imade G.E. et al,[20] demonstrated that the Improved Neubauer Hemocytometer gave the highest CV, while the Makler chamber gave the lowest. In the present study, it could be due to the extreme sperm concentration values obtained from both chambers.

Mahmoud AMA et al,[21] compared 10 different methods for sperm concentration estimation and, as in our study, they found that the Improved Neubauer Hemocytometer gave the best results with the lowest CV. Their study also revealed that the Makler chamber significantly overestimates sperm concentration. Lu JC et al,[17] also had a similar finding in their study, when they compared the ranges of CV between the two chambers for different mean concentrations.

After placing the cover glass on the semen droplet, which is placed on the base of the Makler chamber, there occurs a specific motion & flattening of the fluid which leads to uneven distribution of sperms. This could be the reason for the higher sperm concentration obtained by the Makler chamber.[23]

The Improved Neubauer Hemocytometer is recommended as the current "gold standard" by the WHO manual and considered as standard for sperm concentration measurement.[2] However, the reliability of this method has been challenged.[17] Makler, in 1978, introduced the Makler chamber which was designed specifically for undiluted semen to decrease variations produced by the Hemocytometer.[24]

The Improved Neubauer Hemocytometer method requires multiple steps beforehand, a well experienced examiner, and the use of standardized techniques. All these make the use of this chamber complicated and time consuming. It cannot be used to analyse sperm motility either.

The Makler chamber, being 10 µm deep, allows for the measurement of both sperm count and motility, easily and rapidly in a single step with an undiluted sample. Additional instruments and procedures like pipettes, diluting fluids, pipette flushing, and drying are not required. Despite that, this chamber lacks precision as the minimum number of sperms that can be counted are not less than 1 million/ml. Therefore, we contemplated using the Makler counting chamber as a tool for the initial assessment for infertility and the Improved Neubauer Hemocytometer for a more precise sperm concentration measurement for epidemiological studies, in research or fertility prognosis because precise data is essential.

CONCLUSION

As we found a significant difference between the sperm concentrations measured by the Makler chamber and the Improved Neubauer Hemocytometer for the same semen samples, we conclude that the Makler chamber cannot be used in laboratories for routine semen analysis performed for infertility diagnosis even though it might be done more easily and rapidly.

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