



## Semen Leucocytes Affect Sperm Quality of Infertility Patient

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**Abstract:** The association between the risk factors for male infertility including smoking, obesity, male age, and leukocyte count with sperm analysis, still shows mixed results. This study aims to determine the association between smoking, obesity, male age, and leukocytes count with sperm quality (sperm concentration, sperm motility, and sperm morphology) of infertility patients in Purwokerto. This study is an observational study with a cross-sectional design conducted in the medical records section of the Bunda Arif Hospital Purwokerto. The sample was taken by total sampling. The bivariate test of smoking and obesity behaviour variables with the results of sperm quality using the Chi-Square test and Fisher's exact test. Male age variables used the Kruskal-Wallis test and the Spearman correlation test. Variable leukocytes count using the Spearman test. The results showed no association between male age, obesity and smoking behaviour with sperm quality, sperm concentration, sperm motility, and sperm morphology ( $p > 0.05$ ). There is a significant association ( $p < 0,05$ ) between leukocyte semen count and spermatozoa concentration, sperm motility, and morphology of spermatozoa. It is concluded that there was a significant association between leukocyte count and sperm quality in infertility patients in Purwokerto. Research needs to be continued by examining the relationship between leukocytopenia and sperm DNA damage by looking at sperm DNA fragmentation.

**Keywords:** Infertility; leukocytes count; male age; obesity; sperm analysis; smoking behavior.

### INTRODUCTION

Infertility is the failure to achieve pregnancy clinically after 12 months or more with regular sexual intercourse without contraception (WHO, 2020a). In 2019, the infertility rate in Indonesia was 21,3%, while Central Java's was 5,5%. Furthermore, about 30% of infertility is caused by the female factor, 30 % male factor and the rest is a combination of both (Harzif, Santawi and Wijaya, 2019). Male infertility is characterized by semen-release dysfunction or sperm abnormality inside the semen. The diagnosis was taken by anamnesis, physical examination, additional supporting examination and essential laboratory examination for early evaluation by sperm analysis (WHO, 2020b).

Male infertility risk factors are age, tobacco smoking and obesity (Harzif & Wiweko, 2019). The aging process disrupts the male reproductive system by a hormonal shift in the hypothalamus-hypophysis-testis axis that is characterized by the elevation of FSH and LG levels, disruption of testis histology, reduction of Sertoli cells and Leydig cells, degeneration of germinal cells (Pino et al., 2020) and sperm vulnerability to reactive oxygen species (ROS) compared with younger male (Koh et al., 2016). Tobacco chemical substances induce free radicals-antioxidants imbalance, and oxidative stress interferes with the quality and quantity of sperm function and leads

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to infertility (Franzoni et al., 2021). Male obesity with BMI  $\geq 25$  kg/m<sup>2</sup> impairs spermatogenesis and sexual dysfunction by reduction of testosterone and sex hormone binding globulin (SHBG). Testosterone reduction impairs the physical structure and molecular structure of testis germinal cells reducing the quality and quantity of sperm (Ferial, 2016). Sperm analysis had many parameters, one of them was sperm leucocytes, which correlated with spermatozoa quality, sperm motility impairment, reducing transport and sperm endurance in female reproductive ducts (Sengupta et al., 2022) and by ROS pathway has a role in decreased semen quality (Harzif & Wiweko, 2019).

In Sperma analysis, the correlation between infertility risk factors such as tobacco smoking, obesity, age, and leucocytes has many variations of results. Leukocytospermia is often found in infertile men than in fertile men (Fedder, 1996). However, a meta-analysis that evaluated the impact of leukocytospermia in men attending a fertility clinic showed no association between the condition and reduced fertility after ART and altered semen quality (Castellini et al., 2020). Kumar et al. 2017 found a decrease in sperm quality and quantity parameters in infertile men over 35 years in India, but the Ilyassa., 2019 study gave other results that there was no influence of age with the results of semen analysis of infertile men in Palembang. The research of Hassa et al., 2006 obtained the results that the concentration, motility and morphology of spermatozoa between smoking and non-smoking men were not significantly different, while the study of De Brucker., et al. 2020 found that there was a decrease in semen volume and spermatozoa concentration in smoking men compared to non-smoking men. It can be concluded that the effect of age, smoking habits, and leukocytospermia on infertility in men are still unclear and inconsistent.

Due to the inconsistent research results and the absence of studies that explain the relationship between leukocytospermia and sperm quality and have never been taken in Purwokerto Indonesia, this study is expected to prove the effect of age, smoking habits and sperm leucocytes on male infertility.

## **MATERIALS AND METHODS**

This is retrospective observational research with a cross-sectional design—subjects taken with total sampling technics in medical records, matched with inclusion and exclusion criteria. Inclusion criteria are male with an infertility diagnosis, 20-50 years old, body mass index (BMI) measurement data, tobacco smoking, consent to analyze sperm manually by WHO methods, and leucocytes in semen in sperm analysis data. Exclusion criteria were a history of endocrine disease, chromosome disease, autoimmune disease, history of anatomic impairment, and incomplete data in the medical record. This research was conducted at the Medical Records and Supporting Laboratory of RSIA Bunda Arif Purwokerto Indonesia using secondary data from medical records and sperm analysis results. Samples were taken using the total sampling method on infertility patients who underwent a WHO manual sperm analysis examination for January 2019 - August 2020 and obtained 94 patients that met the inclusion criteria.

Methods for semen analysis were performed according to WHO recommendations (WHO., 2021). Semen samples were collected in the hospital by masturbation into a sterile container after 3–4 days of sexual abstinence. Routine semen analyses include sperm volume, pH, count, progressive motility and morphology—methods for assessing leucocytes in semen using the peroxidase test recommended by WHO (WHO., 2021). A stock solution was obtained by mixing 50 ml 96% ethanol with 50 ml distilled water and adding 125 mg benzidine (Sigma, Milan,

Italy). The working solution was assayed by adding 5 µl 30% H<sub>2</sub>O<sub>2</sub> to 4 ml of stock solution. Twenty µl of liquefied semen were mixed with 20 µl of working solution in a small test tube. After incubation for 5 min at room temperature, 20 µl of the working solution was mixed with 20 µl of phosphate-buffered saline and 10 µl were placed in a haemocytometer, and counted the number of peroxidase-positive cells, the dark brown round cells.

The data is secondary data, taken in medical records, including age, body weight, body height, smoking habit, semen leucocyte quantity, spermatozoa concentration, spermatozoa motility and morphology. The univariate analysis describes research variables by number (n) and percentage (%). In contrast, the bivariate analysis uses Chi-Square or Fisher test as an alternative to examining the correlation between smoking habit and obesity as sperm analysis. Bivariate analysis between male age and sperm analysis results uses Kruskal-Wallis and Spearman correlation test between semen leucocyte quantity and sperm analysis sperm. The research was ethically approved by the ethical commission of medical faculty Jenderal Soedirman university number 200/KEPK/X/2020 on 26th October 2020.

**RESULTS AND DISCUSSION**

Data was collected from Bunda Arif Hospital Purwokerto Indonesia medical records between January 2019 until Agustus 2020. The sample is 94 respondents being grouped by variables, with ages between 23-44 years old, with mean age is 30,87 years old in reproductive ages, mean semen leucocytes quantity is 0,36 million/ml and mean BMI is 23,97 kg/m<sup>2</sup>. Spermatozoa concentration data showed 72.2% of respondents had average and 27.8% abnormal spermatozoa concentrations, normal spermatozoa motility was 87.3% and 12.7% abnormal, and spermatozoa morphology was 17.7% with normal morphology and 82 .3% abnormal.

Kruskal-Wallis tests between age with sperm analysis (concentration, motility, and morphology) in table 2 have p-value > 0,05 means no significant effects of age and sperm morphology between groups (29 years, 30-35 years, 36-40 years and >40 years). Spearman correlation test has p-value = 0,658 for sperm concentration, p = 0,397 for motility, and p=0,567 for morphology. It means no significant correlation between age and the sperm analysis results. According to the statistic test in table 3, there is no significant correlation between IMT with spermatozoa concentration, motility and morphology (p >0,05). Thus, there is no significant correlation between obesity and spermatozoa concentration, motility and morphology.

Table 1. Research Characteristic Distribution

Variable (N=62)	Mean ± SD	Median	Min	Max
Age (years)	32,45 ± 6,04	31,50	23	49
Body weight (kg)	69,30 ± 12,33	68,00	39	104
Body height (m)	1,69 ± 0,04	1,70	1,58	1,83
BMI (kg/m <sup>2</sup> )	23,97 ± 4,05	23,25	15,23	35,57
Sperm concentration (million/ml)	34,28 ± 31,45	26,50	3,70	187,50
Sperm motility (%)	69,56 ± 17,03	73,00	29	136
Sperm morphology (%)	4,79 ± 13,68	2,00	0	89
Sperm leucocytes quantity (million/ml)	0.36±0,42	0,3	0,01	2,7

Table 2. Kruskal-Wallis Male Age Results with Sperm Analysis

	Male Ages	N	Mean Rank	Asymp. Sig.
Spermatozoa Concentration	Group 1	37	46,16	0,616
	Group 2	34	45,68	
	Group 3	15	51,67	
	Group 4	8	53,63	
	Total	94		
Spermatozoa Motility	Group 1	37	49,35	0,443
	Group 2	34	44,38	
	Group 3	15	49,27	
	Group 4	8	48,88	
	Total	94		
Spermatozoa Morphology	Group 1	37	50,19	0,522
	Group 2	34	45,71	
	Group 3	15	47,73	
	Group 4	8	42,25	
	Total	94		

Table 3. Bivariate Analysis: Chi-Square and Fisher Test of Obesity and Sperm Analysis

	Sperm Analysis Parameter	N	P value ( <i>Chi-Square</i> )	P value ( <i>Fisher</i> )
IMT	Spermatozoa concentration	62	0,574	
	Spermatozoa morphology	62		0,748
	Spermatozoa motility	62		0,661

Table 4. Bivariate Analysis: Chi-Square of Smoking Habit and Sperm Analysis

	Non-smoking		Smoking		Total	P-value
	N	%	N	%		
Concentration						
Normal	30	81,1	27	64,3	57	0,097
Abnormal	7	18,9	15	35,2	22	
Motility						
Normal	30	81,1	39	92,9	69	0,176
Abnormal	7	18,9	3	7,1	10	
Morphology						
Normal	5	13,5	9	21,4	14	0,358
Abnormal	32	86,5	33	78,6	65	
Total	37	100	42	100		

The Chi-square test between smoking habit and spermatozoa habit (table 4) has p-value = 0,097 ( $p > 0,05$ ), so there is no significant correlation between smoking habit with spermatozoa concentration. We use the Fisher test as an alternative test for spermatozoa motility variables because the chi-square requirement is not fulfilled with a p-value of 0,176 ( $p > 0,05$ ). It means there is no significant correlation between smoking habits and spermatozoa motility. Chi-square for spermatozoa morphology has a p-value of 0,358 ( $p > 0,05$ ), so there is no significant correlation between smoking habit and spermatozoa morphology.

Spearman test in table 4 shows a significant correlation between semen leucocyte quantity with spermatozoa concentration, motility, and morphology with p value 0,00 ( $p < 0,05$ ). Correlation power between semen leucocytes quantity with sperm analysis are: spermatozoa concentration has a strong correlation ( $r = 0,628$ ), spermatozoa motility has a pretty strong correlation ( $r = 0,401$ ), and spermatozoa morphology has a strong correlation ( $r = 0,58$ ).

Table 5. Bivariate Analysis: Spearman Test of Semen Leucocytes Quantity and Sperm Analysis

	Sperm Analysis Parameter	N	p-value	r
Semen Leucocytes Quantity	Spermatozoa concentration	84	0,00	0,628
	Spermatozoa motility	84	0,00	0,401
	Spermatozoa morphology	84	0,00	0,580

This research shows no correlation between male age, smoking habit, and obesity with sperm concentration. Sperm concentration is defined by dividing the total spermatozoa number by semen volume, so spermatozoa concentration cannot be affected if only the semen volume is reduced. The mean age data was  $32.28 \pm 7.06$  years, ranging from 23 to 75 years, and the highest number of infertility patients was in the 29-year age group (table 1). The Kruskal-Wallis test showed that there was no difference in the effect of age and sperm morphology ( $p > 0.05$ ) (Table 2). This research has a lot younger male respondents than older, and the data showed that in age  $> 40$ , the abnormal sperm concentration percentage is higher than in the younger group. Thus, a degenerative process in older males still occurred but could not represent the population because younger male respondents are more dominant (Zhang et al., 2019).

This study follows the research of Park et al. (2014), which showed that sperm quality and quantity were not related to age but could be caused by other factors such as varicoceles, extreme temperatures, genetic defects, toxic substances and environmental exposures. Age is associated with a decline in semen volume, sperm total, progressive motility, normal sperm morphology, and increased DNA fragmentation. However, despite its decline over time, sperm concentration did not decline with increasing male age. These age-dependent changes in semen quality are attributed to regular physiological changes in the reproductive tract that occur with ageing, as the testis undergoes age-related morphological changes such as a decrease in the number of germ cells, Leydig and Sertoli cells and structural changes, such as the narrowing of seminiferous tubules (Durairajanayagam, 2018).

The average body mass index (BMI) of respondents was  $23.97 \pm 4.05$ , which was categorized as normal BMI with non-obese (62.9%) and obese (37.1%) patients. The Chi-Square test showed that there was no relationship between body mass index and spermatozoa concentration (p-value = 0.574 >0.05), spermatozoa morphology (p-value = 0.748 >0.05), and spermatozoa motility (p-value = 0.661p >0.05) (table 3). This research is similar to (Kheradmand et al., 2017), that concluded that there were no significant differences in normal BMI, overweight and obesity groups related to spermatozoa motility, spermatozoa morphology and semen volume.

This could be because research subjects are rarely randomly selected in the general population due to low participation rates (below 30%), not measuring BMI accurately but only reported by respondents, causing biased associations. Using only a single sperm quality variable without adding additional tests such as hormone tests (testosterone, LH, FSH, and estradiol) so that it is not sufficient to represent male testicular function at any given time, the relatively small sample size may not provide sufficient statistical power to detect an association significantly. Differences in baseline characteristics of enrolled samples in different studies, such as ethnicity and sample proportion of obesity, study design and variations in inclusion and exclusion criteria between different studies, may result in heterogeneity between studies (Kheradmand., 2017).

Overweight (BMI 25–30 kg/m<sup>2</sup>) and obese (BMI >30 kg/m<sup>2</sup>) in males are associated with a decrease in sperm quality and a greater risk of infertility, the prevalence of sperm concentration, total sperm count and low ejaculate volume increased. However, there is no association between body size and sperm motility, morphology or DNA damage. The presence of excess white adipose tissue in obese individuals causes the increased conversion of testosterone to oestrogen. It affects the HPG axis leading to a reduction in gonadotrophin release and impaired spermatogenesis. Increased leptin production by the white adipose tissue decreases testosterone production, scrotal adiposity causes heat stress in the testis and causes oxidative stress, and scrotal temperature raises impairs spermatogenesis, sperm motility and DNA integrity (Durairajanayagam, 2018).

Data showed that 46.8% of respondents do not smoke, and 53.2% are active smokers. The results of the chi-square test showed that smoking was not associated with spermatozoa concentration p-value = 0.097 >0.05), spermatozoa motility (p-value = 0.176 >0.05), and spermatozoa morphology (p-value = 0.358 >0.05) (table 4). This study is by Harlev et al. (2015), whose hypothesis is that; The fertility measurement parameters used to assess the effect of smoking on different, different study designs in each study, differences in population types, and inadequate methods to measure smoking exposure and confounding variables such as age, occupation, drug use, alcohol consumption exposure, history disease, ethnicity and race, socioeconomic status and hormonal changes

The other study showed that sperm concentration in male smokers lower 13–17% lower than in non-smokers, but cigarette smoking has been negatively associated with sperm count, morphology, and motility. The decline in semen quality was found in moderate (10–20 cigarettes/day) and heavy (>20 cigarettes/day) smokers, and the effect size was higher in infertile males. While in this study, we did not ask how many cigarettes were consumed daily. Impairment of semen quality amongst male smokers has been connected to decreased expression of checkpoint kinase 1 (Chk1). Without Chk1 activated in response to DNA damage, there would be a decline in sperm repair leading the sperm apoptosis rised, that made lowering semen quality (Gonzalez et al., 2015). Smoking is associated with leucocytospermia, a primary endogenous source

of reactive oxygen species (ROS). Increased seminal levels of ROS exposed spermatozoa to oxidative stress, caused consequently impairing sperm function (Durairajanayagam, 2018).

The mean number of leukocytospermia in the study was 0.36 million/ml, categorized as an average leukocyte count. Spearman test showed a significant relationship (p-value of 0.00 <0.05). The number of semen leukocytes and the concentration of spermatozoa, spermatozoa motility, and spermatozoa morphology strongly correlated (table 5). These results are in line with research by Lackner et al. (2010), who explained that there was an increase in the progressive motility of spermatozoa in semen samples with leukocyte counts

Leukocytospermia can impair spermatogenesis and sperm maturation by altering cytokine levels, which, in turn, impairs Sertoli cell function, generate reactive oxygen species (ROS) that can disturb sperm motility, hinders the fertilization potential of spermatozoa by interfering with the acrosome reaction and the fusion of sperm and egg, and produced high concentrations of ROS and interferon- $\gamma$ , which can inhibit sperm function and decreased the rate of IVF. Due to this, the presence of leukocytes in seminal plasma was considered a significant prognostic factor for failed embryo transfer and in vitro fertilization. Leukocytospermia-induced DNA fragmentation and sperm DNA fragmentation have been implicated as essential factors in male infertility, so the leukocytospermia examination can be used for initial screening for the success of in vitro fertilization in clinical laboratories with limited facilities. Research needs to be continued by examining the relationship between leukocytospermia and sperm DNA damage by looking at sperm DNA fragmentation (Khodamoradi et al., 2020).

The limitations of the study are a small number of respondents, retrospective method and confounding factors that are not controlled, such as habits, hormonal factors, type of work, length of the marriage, length of experience with infertility, abstinence, and a history of diseases that affect fertility (Oliveira et al., 2018). The secondary data of the study found that the age of respondents who were >40 years old, even though the age of 40 years, was known as the age limit for the start of the male reproductive aging process so that it could affect the results of the analysis. Data on the characteristics of smoking duration, number of cigarettes per day and types of non-existent cigarettes can also affect the study's results (Jeng et al., 2014).

## CONCLUSION

No significant correlation between smoking habit, obesity, and male age with sperm analysis results (concentration, motility, and morphology). There is a significant correlation between semen leucocyte quantity with spermatozoa concentration, motility and morphology in infertility patients in Purwokerto. Researchers suggest conducting additional research to control confounding variables such as age, body weight, marriage duration, program duration, abstinence, drug usage, disease history and other factors affecting infertility. Next, research should clarify tobacco smoking quantity, smoking duration, and type of smoke, and also, the sperm analysis should be taken in the same laboratory to minimize data heterogeneity.

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### CONFLICT OF INTEREST

All authors state no conflict of interest in the publication's writing.

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