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The Impact of Smoking on Sperm Quality and the DAZ Gene

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Abstract: Indonesia's male population is mostly smokers. The chemical content in cigarettes can reduce the quality of spermatogenesis, where spermatogenesis is influenced by the DAZ gene (deleted in Azoospermia). This study aims to analyze sperm quality in light, moderate, and heavy smokers in 3 age groups and analyze the presence of the DAZ gene in heavy smokers. This study used 180 samples divided into three age groups, namely men aged 26-30 years, 31-35 years, and 36-40 years. Each age group recorded the number of cigarettes consumed per day (light, moderate, and heavy smokers) and examined the quality of sperm. While the DAZ gene analysis used 10 samples of heavy male smokers, aged 25-40 years. Sperm quality analysis was based on the WHO laboratory manual for human semen testing, while DAZ gene analysis used the PCR method. Sperm quality data were analyzed using Kruskal-Wallis, while the DAZ gene was analyzed qualitatively descriptively by analyzing electrophoresis photos. The results of this study can be concluded that daily cigarette consumption in the age group affects sperm quality and in heavy smokers, there are 10% who experience deletions in the DAZ gene. Thus cigarettes can reduce spermatozoa quality.

Keywords: Cigarette; Gene DAZ; Quality sperm; Smokers

Introduction

Indonesia is ranked the fifth largest tobacco producer in the world. According to the Ministry of Industry, the amount of tobacco production in Indonesia ranges from 190,000-200,000 tons per year. Indonesia is also the second largest tobacco consumer in the world after China. It is estimated that around 65% of Indonesian men are smokers, while women are much lower, around 3%. According to the World Health Organization, around 22.3% of the world's population used tobacco in 2020. This includes 36.7% of men and 7.8% of women. In high-income countries, 22% of adults are smokers, while in middle-income countries, the figure is 19.5% (Dai et al., 2022). The prevalence of smoking among Indonesian adolescents aged 13-15 years is around 19.2%. The burden of disease in Indonesia is increasing every year. Tobacco use is estimated to be the leading cause of death for smokers, with around 225,700 people dying prematurely, or around 15% of all deaths (Martini et al., 2022). The prevalence of smokers in Indonesia varies by age and gender, namely in 2021, 62.9% of men and 4.8% of women aged 15 years and over were tobacco users. The proportion of daily active smokers aged ten years and over was 33.4% in 2013, then decreased in 2018 to 24.3% (Holipah et al., 2020). Based on a survey of regular smokers in Indonesia in 2019, 45% of male respondents admitted to having tried smoking for the first time before the age of 15 (Kusumawardani et al., 2018). Overall, the prevalence of smoking in Indonesia is relatively high, especially among men and adolescents. The average number of cigarettes smoked per day in Indonesia varies.

Based on a survey of regular smokers in Indonesia in 2019, 32% of respondents admitted to smoking

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between ten and 15 cigarettes per day. When asked about "Cigarettes smoked per day", 24% of Indonesian respondents answered "1 to 5 cigarettes. It is estimated that an Indonesian smoker spends around 5-7 percent of their monthly income to buy cigarettes or other tobacco-related products (Djutaharta et al., 2022). Thus, smoking is common in Indonesia, with the majority of the male population smoking regularly.

The negative impacts of smoking on health are increasing the risk of lung cancer, heart disease, stroke, respiratory disease, and heart disease (Nargis et al., 2022). In the economic field: causing significant health costs, including medical costs and lost productivity due to disease and premature death (Ekpu & Brown, 2015). The tobacco industry may provide some economic benefits, such as jobs or tax revenues, but the real impact of smoking far outweighs any benefits. Cigarettes contain numerous toxic compounds, including tar, nicotine, nitrosamines, carbon monoxide, PAH compounds, and others, which are harmful to the body. The toxic content of cigarette smoke, particularly PAH, has been linked to testicular atrophy, impaired spermatogenesis, and damage to sperm morphology (Mendel et al., 2018; Soleimani et al., 2022; Zeng et al., 2022).

Cigarette smoke contains free radicals, which are atoms or groups of atoms with unpaired electrons that can cause oxidative damage in the body. Free radicals in cigarette smoke are thought to contribute to the development of smoking-related diseases. Regarding free radicals produced by cigarettes, cigarette smoke contains two different populations of free radicals, one in the tar phase and one in the gas phase. The tar phase contains several relatively stable free radicals, while the gas phase contains highly reactive free radicals. The levels of gas-phase radicals in cigarette smoke vary widely between cigarette brands, while particulate-phase radicals show less variability. Free radicals, also known as reactive oxygen species (ROS), can cause oxidative stress in the male reproductive tract when there is an imbalance between free radical formation and antioxidant capacity. Increased ROS levels are seen in 30-80% of men with male infertility (Hussain et al., 2023).

Free radicals can cause sperm dysfunction, leading to specific diagnoses and cases of idiopathic male infertility (Agarwal et al., 2014). A certain amount of free radicals is required for normal sperm function, but excessive levels of free radicals can cause adverse effects on sperm function. Oxidative stress caused by free radicals has been shown to affect standard semen parameters and fertilization capacity (Dutta et al., 2021). High levels of free radicals can reduce sperm motility and cause damage to sperm membranes and mitochondria. A certain amount of free radicals is required for normal sperm function, but excessive levels of free radicals can cause adverse effects on sperm function, fertilization, and offspring health (Agarwal et al., 2014).

Oxidative stress caused by free radicals can have a significant impact on smokers, regardless of the number of cigarettes smoked. Increased serum malondialdehyde (MDA) levels and decreased paraoxonase (PON1) activity may be important in determining the oxidant/antioxidant imbalance in smokers. Smokers have increased levels of oxidative stress and inflammatory biomarkers compared to nonsmokers. Metals in tobacco smoke are also major contributors to oxidative stress in smokers. The impact of oxidative stress on smokers is independent of the number of cigarettes smoked, even light smokers can experience oxidative stress (Caliri et al., 2022).

Smoking significantly reduces semen volume, sperm viability, sperm motility, sperm morphology, and sperm concentration (Bannison et al., 2016). The number of cigarettes smoked per day did not significantly affect semen volume, lethargy, PC, EC, and RBC, but significantly decreased semen pH, sperm motility, viability, morphology, number, and total sperm count (Dai et al., 2015). Inhalation of cigarette smoke causes the absorption of nicotine, carbon monoxide, and heavy metals throughout the body, which can end up in the smoker's seminal plasma through various diffusion and active transport routes (Rehman et al., 2019). Severe DNA damage due to smoking is associated with abnormal spermatozoa and male infertility. Progressive sperm motility in the moderate and heavy smokers group decreased significantly, compared to non-smokers, while there was no significant change in the light smokers group (Cui et al., 2016). Severe DNA damage from smoking is associated with abnormal spermatozoa and male infertility (Huang et al., 2021). DNA damage from cigarette smoke can cause mutations and chromosomal abnormalities in sperm, which can affect fertility and increase the risk of birth defects and genetic disorders in offspring (Yamaguchi, 2019).

The DAZ genes are a family of genes encoding RNA-binding proteins essential for gametogenesis in metazoans. They are expressed at all stages of germline development and are essential for germline development. Deletions of the DAZ genes occur in 10% of males with spermatogenic defects, and infertile males who exhibit loss of a copy of the DAZ gene are highly susceptible to azoospermia or severe oligozoospermia. However, due to the presence of a functional homolog (DAZLA) on human chromosome 3, a direct association between DAZ gene deletion and azoospermia has been difficult to establish. Studies have also identified a single nucleotide polymorphism in DAZL that confers susceptibility to spermatogenesis defects. Although copies of the DAZ gene are deleted in males with

complete and partial AZFc deletions, individual copies of DAZ can be deleted or duplicated even in the absence of complete deletions. In conclusion, the DAZ genes play an important role in male fertility, and deletions of these genes are associated with spermatogenic defects and infertility (Rosario et al., 2016).

The prevalence of DAZ gene deletions in infertile men varies depending on the population studied and the type of spermatogenic disorder. A study conducted in a Southern Chinese population found that deletion of DAZ1/DAZ2 was associated with spermatogenic disorders, while deletion of DAZ3/DAZ4 had little or no effect on fertility (Colaco & Modi, 2018). Another study found that the frequency of DAZ microdeletions was estimated at 11.1% in azoospermia and 6.25% in oligozoospermia. DAZ deletions occur in 10% of men with spermatogenic defects, and infertile men who show loss of a copy of the DAZ gene are highly susceptible to azoospermia or severe oligozoospermia (Liu et al., 2022). Cigarette smoke has been shown to increase the frequency of Y chromosome microdeletions, including DAZ gene deletions. The following are some of the effects of cigarette smoke on the prevalence of DAZ gene deletions: Cigarette smoke contains mutagens that can cause DNA deletions.

A study found that the frequency of Y chromosome microdeletions was higher in smokers than in non-smokers. Adult male mice exposed to tobacco smoke showed a significant increase in sperm DNA mutations at expanded simple tandem repeats. In conclusion, cigarette smoke can increase the prevalence of DAZ gene deletions by causing DNA deletions, increasing the frequency of Y chromosome microdeletions, and altering sperm DNA methylation patterns (Dutta, 2023). This study aims to analyze the sperm quality of light, moderate, and heavy male smokers in various age groups and to analyze the presence or absence of DAZ gene deletion in heavy male smokers.

Method

Sperm Quality

Subject/Patients: Semen from 180 men who came to the andrology laboratory, Dr. Sutomo hospital in Surabaya, which was divided into 3 groups, namely group A consisting of 60 people aged 26-30 years, group B consisting of 60 people aged 31-35 years, group C aged 36-40 years, 60 people. Each group was divided into 4 subgroups with each subgroup consisting of 15 people, namely A1, B1, C1: light smokers, D1: control. A2, B2, C2: moderate smokers, D2: control. A3, B3, C3: heavy smokers, D3: control. Criteria for light smokers: less than 10 cigarettes/day, moderate smokers: 10-20 cigarettes/day, heavy smokers: 20-30 cigarettes/day. Control: non-smoking men.

Semen Analysis

Patients who meet the inclusion criteria, ejaculate sperm by masturbation and collect it in a wide-mouthed glass cup. Labeled with patient code, date, and time of ejaculation. Liquefaction, pH, volume, viscosity, motility, morphology, viability, and concentration were analyzed according to the WHO laboratory manual for the examination and processing of human semen.

Liquefaction

Semen that has just been ejaculated is usually thick, at room temperature it will liquefy within 15-20 minutes. Observations are made for one hour, with occasional stirring, liquefaction of more than one hour needs to be reported. After liquefaction and complete liquefaction, the sample is ready to be processed. pH checked within one hour after ejaculation by placing one drop of sperm on pH paper and leaving it for 30 seconds, then compared to the comparison standard. Normal pH ranges from 7.2 to 8.0.

Volume

Measured using a measuring cup by transferring all the sperm fluid into the measuring cup, and then reading the scale on the measuring cup. Normal volume ≥ 1.5ml.

Viscosity

Measured using an Eliasson pipette by taking sperm using an Eliasson pipette to the limit mark, then allowed to drip by itself according to gravity. calculated how long it takes to drip. Normal sperm has a viscosity of 2 cm.

Motility

Calculated immediately after perfect liquefaction by placing one drop of sperm on a glass object then covering it with a cover glass and observing it. Motility is calculated based on the WHO criteria, with grades a. fast and straightforward movement, b. forward movement, but not fast enough, c. movement in place, d. not moving. It is said to be normal if motility a+b ≥50%, or a ≥25%.

Morphology

Place one drop of sperm on a glass object, then make a sperm smear. Allow to dry at room temperature. After drying, the preparation is fixed using methanol for 5 minutes, then dried. After the preparation is fixed and dry, it is then stained with safranin for 5 minutes. Then washed using phosphate buffer and stained with crystal violet for 5 minutes. Then washed using water. Observations are made using a microscope with a magnification of 1000X. Observed spermatozoa with normal and abnormal morphology.

Viability

Viability is calculated between live and dead spermatozoa. Live spermatozoa when stained with 0.5% eosin are not colored, dead spermatozoa when stained with 0.5% eosin are colored red. It is done by placing one drop of sperm on a glass object then adding one drop of 0.5% eosin, then homogenizing, leaving it for 2 minutes, then observing, it is said to be normal if the live spermatozoa are ≥ 60%.

Concentration

The concentration test begins by estimating the density of sperm in a wet preparation, which is used to determine the dilution factor as follows: For preparations with the number of sperm per field of view (400X), < 15 spermatozoa, the dilution is 1: 5. For preparations with the number of sperm per field of view (400X), < 15 - 40 spermatozoa, the dilution is 1: 10. For preparations with the number of sperm per field of view (400X), < 40 - 200 spermatozoa, the dilution is 1: 20. For preparations with the number of sperm per field of view (400X), > 200 spermatozoa, the dilution is 1: 50.

$$C = \frac{\text{N x 10.000 x diluent factor x 25}}{\text{number of boxes counted}} \quad (1)$$

DAZ Gene

Sample: semen of 10 active male smokers aged 25-40 years with a cigarette consumption of 20-30 cigarettes or more per day. DNA Extraction Reagent Materials (Qiagen Blood and Tissue kit), 2x Master mix (Intron), forward primer 5'GGG TGT TAC CAG AAG GCA AA 3' and reverse primer 5' GAA CCG TAT CTA CCA AAG CAG C 3' PCR target 400 bp, agarose gel, DNA marker 100 bp.

Semen Collection

Samples were obtained from the Andrology Polyclinic in Surabaya and male smokers who met the criteria.

DNA Isolation

Put 200 µl of semen sample into a 1.5 ml tube. Add 20 µl of Proteinase K and 180 µl of ATL Buffer, then vortex for 15 seconds and spin down. Incubate for 10 minutes in a thermostat. Add 200 µl of AL Buffer, vortex and spin down. Add 200 µl of 96% Ethanol, then vortex and spin down. All mixtures were put into a spin column. Then centrifuge at 8,000 rpm for 1 minute. Replace the column section with a new one. Take 500 µl of AW 1 then put it into the spin column and centrifuge at 8,000 rpm for 1 minute. Replace the column part with a new one, then take 500 µl of AW 2 and put it into the spin column. Centrifuge at 13,000 rpm for 3 minutes. Replace the column part with a new one then centrifuge again at 13,000 rpm for 1 minute. Move the spin column to a 1.5 ml tube. Insert 50 µl of Buffer AE into the center of the spin column, and incubate at room temperature for 1 minute. Then centrifuge at 8,000 rpm for 1 minute. Discard the spin column part and obtain 50 µl of DNA isolation results (DNA template).

PCR Process

Inserting 12.5 µl 2x Master mix (Intron), 0.5 µl distilled water, 1 µl forward primer, 1 µl reverse primer, and 5 µl DNA template. Then vortex for 15 seconds and spin down. Insert into the PCR machine with pre-denaturation 94^{0}C for 5 minutes, denaturation 94 ^{0}C for 1 minute, annealing 63 ^{0}C for 1 minute, extension 72 ^{0}C for 1 minute, and final extension 72 ^{0}C for 5 minutes with 35 cycles.

Electrophoresis

Electrophoresis using 2% agarose gel and using redsafe (gel dye) 2 µl per 20 ml TBE (Tris Borate EDTA) 1x. Each gel well is filled with 5 µl of PCR product, and then run for 30-40 minutes with an electric voltage of 110 Volts.

Observation

Observations were made using a UV Transilluminator for the documentation report of the research results.

Data Analysis

Sperm quality: Statistical tests include data normality tests using Shapiro-Wilk, data variance homogeneity tests using Levene, normally distributed and homogeneous data are processed using One Way ANOVA, non-normally distributed and non-homogeneous data are processed using Kruskal-Wallis if the Kruskal-Wallis test results produce a p-value <0.05 then it is continued with the Mann-Whitney comparison test.

DAZ gene analysis: descriptive qualitative analysis by analyzing electrophoresis photos.

Result and Discussion

pH Semen

In the age group 26-30 years and the age group 31-35, there was no effect of daily cigarette consumption on the quality of sperm pH. In the age group 36-40 years there was differed significantly. Semen pH reflects the balance between the pH values of the different accessory gland secretions, especially the alkaline seminal vesicle secretion and the acidic prostatic secretion. pH should be measured after liquefaction preferably after 30 minutes, but in any case within 1 hour of ejaculation because it is affected by the loss of CO_2 that occurs after

production. Excess production of free radicals or reactive oxygen species (ROS) can damage sperm, and ROS has been known to be one of the causes of infertility. It is also known that superoxide anions, hydroxyl radicals, and hydrogen peroxide are some of the main ROS present in seminal plasma (Agarwal et al., 2014).

Volume

In the age group 26-30 years, there was no effect of daily cigarette consumption on the quality of sperm volume. In the age group 31-35, the quality of sperm volume of non-smokers did not differ significantly between light smokers and moderate smokers, light smokers with moderate smokers and heavy smokers, and moderate smokers with heavy smokers. The quality of sperm volume of non-smokers was significantly different from heavy smokers. In the age group 36-40 years, the quality of sperm volume of non-smokers was not significantly different from light smokers, light smokers with moderate smokers and heavy smokers, and moderate smokers with heavy smokers. The quality of sperm volume of non-smokers was significantly different from moderate smokers and heavy smokers. The fluid ejaculates at orgasm, and semen, contains sperm and secretions from the seminal vesicles, prostate, Cowper's glands, and possibly the urethral glands. The average volume of ejaculation is 2.5-3.5 ml after several days of not ejaculating. Smokers show decreased semen volume, sperm count, sperm motility, and sperm viability compared to nonsmokers (Bannison et al., 2016).

Table 1. Average Data of Sperm Quality Analysis Results in Different Age Groups and Cigarette Consumption

Age group	Cigarette Consumption	Semen Parameters							
		Liquefaction (minute)	Ph	Viscosity (second)	Volume (ml)	Concentration (10^6 /ml)	Motility (%)	Morphology (%)	Viability (%)
26-30 years	light smokers	20.33	7.19	2.33	2.51	46.56	27.33	3.60	59.67
	moderate smokers	20.33	7.13	2.40	2.25	32.49	22.67	5.60	49.27
	heavy smokers	20.33	7.16	2.27	1.99	22.07	14.33	2.67	42.67
	Non-smoking	20.67	7.51	2.20	2.70	59.87	35.00	8.27	83.80
	Significance (p)	0.981	0.100	0.873	0.096	0.009	0.001	0.001	0.000
31-35 years	light smokers	20.67	7.19	2.10	2.34	29.39	17.33	4.53	47.47
	moderate smokers	21.00	7.15	2.37	2.29	35.69	15.67	4.00	47.30
	heavy smokers	20.33	7.11	2.30	1.73	21.79	13.00	3.07	47.87
	Non-smoking	21.87	7.38	2.17	3.02	55.37	33.00	7.73	78.67
	Significance (p)	0.735	0.365	0.550	0.012	0.031	0.000	0.022	0.006
36-40 years	light smokers	21.99	7.10	2.26	2.60	36.70	15.66	5.53	53.00
	moderate smokers	21.33	7.09	2.30	1.86	23.67	18.33	5.73	48.53
	heavy smokers	29.00	7.04	2.33	1.88	18.78	14.66	3.33	48.46
	Non-smoking	21.66	7.50	2.20	3.36	45.78	28.66	9.06	79.33
	Significance (p)	0.778	0.039	0.948	0.001	0.078	0.017	0.016	0.058

Concentration

In the age group of 26-30 years, the quality of sperm concentration of non-smokers did not differ significantly from light smokers, light smokers with moderate smokers and heavy smokers, and moderate smokers with heavy smokers. The quality of sperm concentration of non-smokers differed significantly from moderate smokers and heavy smokers. In the age group of 31-35 years, the quality of sperm concentration of light smokers did not differ significantly from moderate smokers, light smokers with heavy smokers, and moderate smokers with heavy smokers. The quality of sperm concentration of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers. In the age group of 35-40 years, there was no effect of cigarette consumption per day on the quality of sperm concentration. The negative impact of smoking on human semen parameters correlates with cigarettes smoked per day and duration of smoking. Smokers showed a decrease in semen volume, spermatozoa count, spermatozoa motility, and spermatozoa survival when compared to non-smokers. In addition, smokers showed an increase in leukocytes in semen, the percentage of oval-headed spermatozoa, and the percentage of deformed-headed spermatozoa with cytoplasmic droplets (Bundhun et al., 2019). The results of semen analysis from patients who smoked showed abnormalities in concentration, followed by

abnormalities in spermatozoa morphology and motility. Sperm quality, especially in heavy smokers, the difference is based on the high level of oxidative stress in semen in heavy smokers compared to light smokers and passive smokers (Rehman et al., 2019).

Motility

In the age group of 26-30 years, the quality of sperm motility of non-smokers did not differ significantly from light smokers and moderate smokers, as well as moderate smokers and heavy smokers. The quality of sperm motility of non-smokers differed significantly between moderate smokers and heavy smokers, and the motility of sperm of light smokers differed significantly from heavy smokers. In the age group of 31-35 years, the quality of sperm motility of light smokers did not differ significantly from moderate smokers and heavy smokers, as well as moderate smokers and heavy smokers. The quality of sperm motility of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers. In the age group of 36-40 years, the quality of sperm motility of light smokers did not differ significantly from moderate smokers and heavy smokers, as well as moderate smokers and heavy smokers. The quality of sperm motility of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers.

Sperm damage including that caused by ROS occurs because it can inhibit the acrosome reaction and tail damage which greatly affects spermatozoa motility. Free radicals originating from cigarette gas particles also cause sperm agglutination, resulting in decreased sperm motility. Free radicals are physiologically present in human sperm. The emergence of free radicals in the body is balanced by endogenous defense mechanisms, by producing substances that have an effect as anti-free radicals called antioxidants. However, when the ROS (Reactive Oxygen Species) level exceeds the body's antioxidant defense system, oxidative stress occurs (Durairajanayagam et al., 2019).

Smoking can reduce sperm count and motile sperm count (Pujianto et al., 2021). Sukarjati et al. (2023) reported that Rats exposed to cigarette smoke caused increased MDA levels, decreased spermatogonia, spermatocytes, and spermatids, and decreased seminiferous tubule diameter compared to controls not exposed to cigarette smoke. The mice exposed to electronic cigarette smoke decreased sperm motility, sperm concentration, and viability (Zakiyah & Sukarjati, 2022). According to Engel et al. (2021), nicotine causes a decrease in human sperm motility because smoking can cause sperm cell apoptosis. It is also stated that the number of cigarettes consumed per day and week is significantly related to sperm concentration because cigarette consumption induces oxidative stress in human sperm and sperm apoptosis. ROS can cause infertility through 2 mechanisms, namely (1) damage to the sperm membrane and decreased sperm motility (2) damage to sperm DNA. Two important sources of ROS production in the male reproductive system are immature spermatozoa and seminal plasma leukocytes. Polymorphonuclear leukocytes (PMN) and macrophages produce 50-60% and 20-30% ROS. Leukocytes activated by inflammation and infection produce higher ROS (Asadi et al., 2021).

Spermatocytes are highly susceptible to oxidative stress induction due to the high levels of polyunsaturated fatty acids (PUFA) in their plasma membranes. Lipid peroxidation can cause loss of motility (Nowicka-bauer, 2020). Low levels of antioxidant enzymes such as catalase, or glutathione (GSH) cause spermatocyte damage (Nsonwu-anyanwu et al., 2019). According to Zargari et al. (2022) stated that the biological mechanism of the effect of oxidative stress on sperm function has been identified. This includes lipid peroxidation, DNA damage with indicators of the formation of 8-OhdG, and the occurrence of gene mutations that cause decreased sperm quality (Zargari et al., 2022).

Morphology

In the age group of 26-30 years, the morphological quality of sperm of light smokers did not differ significantly from moderate smokers and heavy smokers, and moderate smokers from heavy smokers. The morphological quality of sperm of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers.

In the age group of 31-35 years, the morphological quality of sperm of light smokers did not differ significantly from moderate smokers and heavy smokers, and moderate smokers from heavy smokers. The morphological quality of sperm of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers.

In the age group of 36-40 years, the morphological quality of sperm of non-smokers did not differ significantly from light smokers, light smokers with moderate smokers and heavy smokers, and moderate smokers with heavy smokers. The morphological quality of sperm of non-smokers differed significantly from moderate smokers and heavy smokers.

Smoking has been shown to have a negative effect on various semen analysis parameters. Several studies have shown that smoking is associated with decreased sperm density, total sperm count, and percentage of motile sperm, as well as increased sperm morphological defects (Kulaksiz et al., 2022). Smoking is associated with lower sperm count and an increased number of sperm morphological defects (Bundhun et al., 2019). Therefore,

there is strong evidence to suggest that smoking may have a negative effect on sperm morphology and other sperm parameters.

Cigarette smoke inhaled by a smoker contains gas and particle components. Gas components have the potential to cause free radicals, including carbon monoxide, carbon dioxide, nitrogen oxides, and hydrocarbon compounds. While the particle components include tar, nicotine, benzopyrene, phenol, and cadmium. The vasoconstrictor effect of nicotine can also cause ischemic conditions in the penis, which also results in abnormalities in spermatozoa morphology. Sperm abnormalities include abnormalities of the head, neck, and tail. The decrease in the number of normal spermatozoa morphology is thought to be caused by excessive ROS production resulting from naturally occurring metabolic products that are harmful to cell survival. ROS can cause lipid peroxides in the spermatozoa plasma membrane which can cause spermatozoa function failure, namely the loss of the ability to fertilize (Agarwal et al., 2022).

Reactive oxygen species (ROS) can have a significant impact on sperm morphology. Increased ROS levels, along with decreased antioxidant defenses, can cause redox imbalance, decreased sperm motility, and sperm DNA damage. ROS can alter DNA integrity in the sperm nucleus, cause DNA strand breaks, and cause chromatin cross-linking, leading to abnormal sperm morphology. In addition, ROS can induce lipid peroxidation in the sperm membrane, which is a major mediator of ROS-induced sperm damage, which further affects sperm morphology and causes infertility. In addition, there is a negative correlation between sperm ROS production and the percentage of normal morphology, indicating a relationship between excessive ROS production and abnormal sperm morphology. Therefore, ROS can have a negative impact on sperm morphology, which can lead to male infertility (Alahmar, 2019).

Smoking has been associated with a lower ability to induce acrosome reaction in smokers' semen samples. An in vitro study showed that benzo (a) pyrene, a component of cigarette smoke, significantly reduced the percentage of acrosome halo formation. In addition, high nicotine levels have been observed to cause premature acrosome reactions in spermatozoa. the induction of acrosome reaction was found to be significantly lower in smokers' semen samples, which may interfere with sperm penetration through the zona and subsequent gamete membrane fusion. These findings suggest that smoking may have a negative effect on the acrosome reaction, which is essential for fertilization (Dai et al., 2015). The destroyed acrosome membrane causes the hydrolytic enzymes contained in the acrosome to come out so that the acrosome cap owned by the spermatozoa becomes incomplete. This is what causes the number of spermatozoa that have intact acrosome caps after exposure to cigarette smoke so that the sperm morphology becomes abnormal. In addition, damage to the spermatozoa membrane causes disorders in the spermatozoa themselves. Damaged plasma membranes cause increased permeability of the cell membrane in the spermatozoa head so that many unwanted compounds can easily enter the cell. Visualization of sperm morphology is presented in Figure 1.

Viability

In the age group of 26-30 years, the quality of sperm viability of light smokers did not differ significantly from moderate smokers and heavy smokers, as well as moderate smokers and heavy smokers.

The viability of sperm of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers. In the age group of 31-35 years, the quality of sperm viability of light smokers did not differ significantly from moderate smokers and heavy smokers, as well as moderate smokers and heavy smokers. The quality of sperm viability of non-smokers differed significantly from light smokers, moderate smokers, and heavy smokers. In the age group of 35-40 years, there was no effect of daily cigarette consumption on the quality of sperm viability.

Oxidative stress is a condition where there is an increase in ROS which will cause damage to cells, tissues, or organs. In oxidative stress conditions, free radicals will cause lipid peroxidation of the cell membrane and damage the organization of the cell membrane. This cell membrane is very important for receptor function and enzyme function so lipid peroxidation of the cell membrane by free radicals can result in total loss of cellular function (Alahmar, 2019).

Oxidative stress through its negative effects on spermatozoa such as increased loss of motility, increased membrane damage, decreased morphology, viability, and ability of spermatozoa (Hussain et al., 2023). The plasma membrane of spermatozoa, especially the tail, has a function to obtain the substrate needed as an energy source and to transmit movement waves. Visualization of sperm morphology is presented in Figure 2.

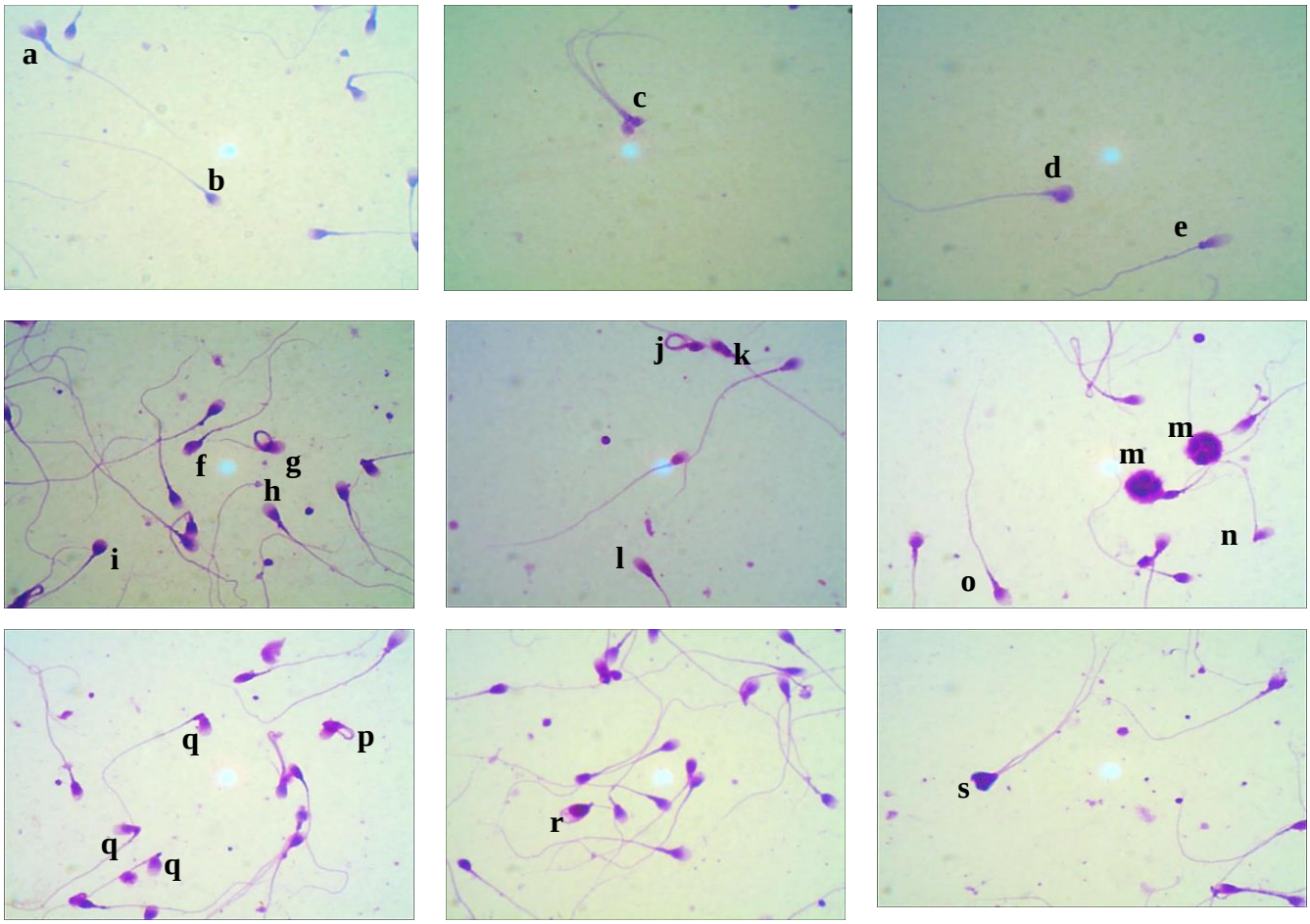
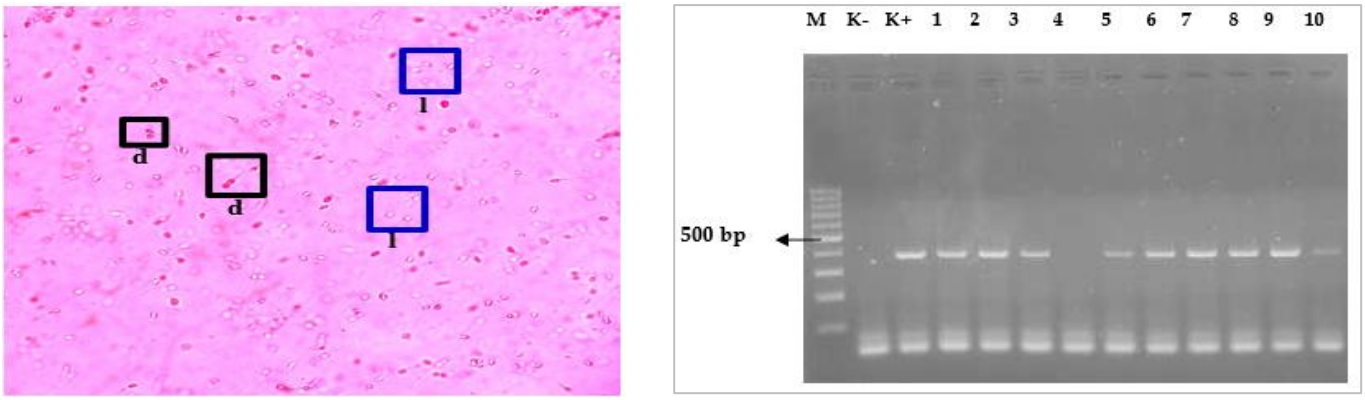


Figure 1. (a) Double head; (b) Round head; (c) Double head triple tail; (d) Round head; (e) Taper head; (f) Amorph head; (g) coil tail; (h) Pin head; (i) Round head; (j) coil tail; (k) Piry tail; (l) coil tail; (m) Leukosit; (n) Bent; (o) Normal; (p) coil tail; (q) Bent; (r) Makro head; and (s) Double head double tail



(a) (b)

Figure 2. (a) Sperm viability: 'd' dead sperm and 'l' live sperm. (b) PCR electrophoresis results of active male smoker sperm sample products. M: DNA Marker 100 bp; K-: Negative control; K+: Positive control; No. 1,2,3,5,6,7,8,9 and 10 positive samples on the 400 bp target; No. 4 negative sample does not show a band on the 400 bp target

Table 2. Effect of Smoking on the Presence of DAZ Gene Deletion

Sample Code	Age (Years)	Status Marriage		Length of Marriage (Years)	number of children	Number of cigarettes consumed per day	Types of Cigarettes	Duration of Smoking (Years)	PCI Results
		Yes	Not						
DAZ 1	27	√	-	5	0	20	White Filter	18	Positive
DAZ 2	40	√	-	12	0	20	White Filter	30	Positive
DAZ 3	27	√	-	2	0	36	White Filter	12	Positive

Sample Code	Age (Years)	Status Marriage		Length of Marriage (Years)	number of children	Number of cigarettes consumed per day	Types of Cigarettes	Duration of Smoking (Years)	PCI Results
		Yes	Not						
DAZ 4	35	√	-	6	0	20	without Filter	16	Negatve
DAZ 5	37	√	-	7	0	20	White Filter	19	Positive
DAZ 6	30	√	-	3	1	20	White Filter	13	Positive
DAZ 7	28	-	√	-	-	24	White Filter	10	Positive
DAZ 8	29	-	√	-	-	20	White Filter	12	Positive
DAZ 9	34	√	-	1	0	20	White Filter	18	Positive
DAZ 10	35	-	√	-	-	20	White Filter	18	Positive

DAZ GENE

A study conducted on 10 sperm samples of active male smokers aged 25-40 years with cigarette consumption of 20-30 cigarettes or more per day to detect deletions in the DAZ gene, as in Table 2.

PCR process was carried out on 10 samples using pre-denaturation 94 ^{0}C for 5 minutes, denaturation 94 ^{0}C for 1 minute, annealing 63 ^{0}C for 1 minute, extension 72 ^{0}C for 1 minute, and final extension 72 ^{0}C for 5 minutes with 35 cycles with a PCR target of 400 bp, electrophoresed with 2% agarose gel and observed using a UV Transilluminator, as in Figure 2 (b).

In this study, active smokers aged 35 years with a cigarette consumption of 20 cigarettes per day and unfiltered kretek cigarettes experienced a deletion in the DAZ gene. This happens because unfiltered kretek cigarettes do not only use tobacco and clove pieces. Kretek cigarettes also contain other dangerous ingredients such as tar and nicotine in quite large amounts. The tar content in Kretek cigarettes is 20 mg and nicotine is 44-45 mg. This is exacerbated by the absence of a filter at the base of the cigarette.

The filter on cigarettes aims to reduce the absorption of harmful substances that enter the body. The filter on cigarettes can absorb 5-15 mg of tar. In the DAZ 6 sample, despite smoking for 30 years and already having a child, the sample did not experience a deletion of the DAZ gene. This is because the type of cigarette consumed is a white cigarette with a filter.

The content of white cigarettes with filters is tobacco pieces and given a certain aroma and taste. White cigarettes with filters contain 14-15 mg of tar, and 5 mg of nicotine, and there is a filter at the base of the cigarette to reduce the absorption of harmful substances as previously explained. While in the DAZ 2 sample who consumed cigarettes with filters but still did not have children, there were several factors causing infertility in men, for example, a. sperm production disorders, namely deletions in the Y chromosome or direct damage related to anatomy (cirrhosis, varicocele), infection (mumps orchitis) or gonadotropins; b. sperm function disorders, namely inflammation of the genital tract (prostatitis) and disorders with sperm adhesion (to the zona pellucida or penetration); c. blockage of the ductus, such as vasectomy, absence of bilateral vas deferens or blockage of the penetrates (Saragih, 2014). Therefore, further research is needed to determine the cause of infertility. DNA damage is an inevitable consequence of cellular metabolism. Normal cell metabolism is an endogenous source of reactive oxygen species and this is the cellular process that explains the background level of oxidative DNA damage detected in normal tissues. The entire electron transport group has the potential to "leak" electrons to oxygen, thus forming superoxide. Oxidative damage to DNA bases occurs due to their reaction with reactive oxygen species. Reactive oxygen species can also be generated by ionizing or ultraviolet radiation. Certain exogenous chemicals in the redox cycle follow cellular metabolism, with the subsequent production of electrons, which can be transferred to molecular oxygen to produce superoxide. Apart from these events, reactive oxygen species can interact with cellular biomolecules, such as DNA, and ROS causing chromatin fragmentation and DNA damage with the result of sperm dysfunction (Bibov et al., 2018). The compound 8-deoxyguanosine is one of the biomarkers of oxidative DNA damage (DNA adduct) (Fenga et al., 2017).

There are several other indicators to determine the occurrence of oxidative stress, for example, Malondialdehyde (MDA) which is a metabolite resulting from lipid peroxidation by free radicals. Malondialdehyde (MDA) can be formed when hydroxyl free radicals such as ROS react with fatty acid components of the cell membrane so that a chain reaction known as lipid peroxidation occurs. This lipid peroxidation will cause the fatty acid chain to break into various toxic compounds and cause damage to the cell membrane. Malondialdehyde (MDA) is a compound that can describe the activity of free radicals in cells so it is used as one of the indicators of oxidative stress due to free radicals (Sulaiman et al., 2024). The process of spermatozoa DNA packaging is unique and has a complex mechanism. This complexity can expose DNA to damage that can occur at any stage. For example, the irregular protamin process results in increased torsional stress, causing additional damage that affects DNA integrity, protein production, and embryo development. Spermatozoa have a limited amount of antioxidants according to the small volume of cytoplasm. This

condition makes spermatozoa susceptible to oxidative stress caused by ROS. In addition, the plasma membrane of spermatozoa is rich in unsaturated fatty acids to maintain membrane fluidity, causing spermatozoa to easily bind to ROS. This mechanism causes oxidative stress as a result of plasma membrane peroxidation, causing damage to spermatozoa and their defense mechanisms (Aitken et al., 2014).

Detection of DAZ gene deletion in male smokers using 10 samples of active male smokers aged 25-40 years with cigarette consumption of 20-30 cigarettes/day or more (Table 2). Detection of DAZ gene deletion using one pair of specific forward and reverse primers with a target of 400 bp. Negative control using distilled water to see whether the work was done correctly. A negative control showed no specific band at the target of 400 bp. A positive control using a sample of fertile male sperm that did not smoke showed a specific target band at 400 bp and was repeated 3 times for valid results. The results of the study in Figure 3 show that of the 10 sperm samples examined, 9 samples were positive for the DAZ gene (90%) and one sample was negative for the DAZ gene (10%).

Negative DAZ gene samples do not show specific bands according to the target indicating the loss of all copies of the DAZ gene. Based on the data in Figure 1, DAZ gene deletion occurred in sample No. 4 with a smoker age of 35 years and smoking 20 cigarettes per day. DAZ is a member of the AZFc gene associated with spermatogenesis and is actively transcribed and translated into human testes. DAZ gene deletion can occur in the meiosis I phase (primary spermatid phase) namely during DNA replication in the spermatogenesis process (Xie, 2020). The DAZ gene in the AZFc region is the most important candidate gene family and is considered to have a diverse role in the spermatogenic process because it is expressed at all stages of germ cell development. This gene regulates translation, encodes a germ cell-specific RNA binding protein, and is involved in meiosis control and maintaining the primordial germ cell population. Deletion of the DAZ gene gives a spectrum of phenotypes ranging from oligozoospermia to azoospermia (Fu et al., 2015).

Various studies have shown that the most frequent deletions involve the AZFc subregion with the strongest candidate gene being DAZ. These data show that the DAZ gene family has been dominant in spermatogenesis compared to other AZF candidates. The main genetic factor associated with the Y chromosome in azoospermic and oligospermic men is 15%. The gene locus on the Y chromosome in azoospermic men is in (AZFa, AZFb, and AZFc) (Punjani et al., 2020). The results of a study conducted by Birowo et al. (2017) reported that microdeletions of the AZF region were not limited to azoospermic men, but were also detected in men with severe oligozoospermia. The results of the study showed that partial deletion of AZFa occurred in 11 men (15.49%), complete deletion of AZF b in 1 man (1.4%), complete deletion of AZFc in 1 man (1.4%) (Birowo et al., 2017). Deletion of the DAZ gene in humans is associated with problems in spermatogenesis ranging from azoospermia to oligozoospermia. Deletion of the DNA copy of the DAZ gene is possible, but cannot be observed using the PCR method alone. It is not possible that 9 sperm samples have deletions in one or more copies of the DAZ gene. Further research is needed to find out more specifically the mutations that occur in the DAZ gene using the DNA sequencing method (Rabinowitz et al., 2021). So that the DNA structure that experiences the DAZ gene mutation can be known.

Smoking without a filter is very dangerous for health, especially for male reproduction. Judging from the results of this study shows that the deletion of the DAZ gene occurs in smokers without a filtered kretek. The dangerous content in cigarettes such as tar, nicotine, and the gas produced has the potential to cause free radicals in the body. Excessive free radicals in the body can damage cell membranes and then bind to DNA bases, causing DNA damage. DNA damage can occur during the spermatogenesis process, namely during DNA transcription so that it can cause deletions in the DAZ gene.

Conclusion

The results of this study can be concluded that daily cigarette consumption in the category of light, 10 cigarettes/day, moderate category, 10-20 cigarettes/day, heavy category, more than 20-30 cigarettes/day in the age group of men aged 26-30 years, 31-35 years, and 36-40 years can reduce sperm quality. Heavy smokers with consumption of Kretek cigarettes (cigarettes without filters) experience deletions in the DAZ gene with PCR method.

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Author Contributions

Conceptualization, S. N and F.; methodology, S. N and F ; software, N and F.; validation, S. N. and F.; formal analysis, N dan F.; investigation, S. N and F.; resources, S. N and F; data curation, S. N and F; writing—original draft preparation, S, N and F.; writing—review and editing, S; visualization, N and F. ; supervision, S; project administration, S,N and F ; funding acquisition, S. N. and F.

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Conflicts of Interest
The Authors declare no conflict of interest.

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