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Solo Black Garlic fermented whey kefir improves sperm quality of male mice (*Mus musculus*) induced by lead acetate

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ABSTRACT

Globally, men experience a decline in total fertility, characterized by a significant decline in sperm quality in terms of concentration, motility, and morphology. Environmental factors can modulate this through exposure to toxic elements, such as the heavy metal lead, in the workplace. Lead can accumulate in nature and contaminate drinking water supply systems. A series of negative responses can occur and disrupt spermatozoa production and quality. Natural ingredients, such as solo black garlic (SBG), have the potential to maintain conditions in reproductive function against the adverse effects of lead. This study utilized SBG fermented with whey kefir to investigate its effect and optimal dosage on improving spermatozoa quality in male mice induced by lead acetate. The test animals were 25 Swiss Webster male mice aged 12-14 weeks, divided into five treatment groups (n = 5 per group), namely negative control (KN), distilled water; positive control (KP), lead acetate 75 mg/kg BW; and three SBG treatment groups given lead acetate (SBG-60, SBG-120, and SBG-180). SBG was given together with lead acetate at doses of 60, 120, and 180 mg/kg BW for 30 days. The results showed SBG-120 restored concentration (31.00×10^6/ml) and motility (80.83%) toward negative-control levels; morphology abnormalities decreased by 30.50%, which were not significantly different ($p > 0.05$) from the negative control. Therefore, SBG with a dose of 120 mg/kg BW is optimum for maintaining spermatozoa quality in male mice exposed to lead acetate.

Keywords: Lead acetate, Solo Black Garlic, spermatozoa, whey kefir

INTRODUCTION

The prevalence of infertility on a global scale currently shows an increasing trend due to lifestyle changes. Infertility is defined as the inability to conceive within one year of active sexual intercourse between the opposite sex without the use of contraception (Huang et al., 2023). The global total fertility rate (TFR) has decreased by more than half, from 4.84 to 2.23, between 1950 and 2021. This trend is projected to continue declining between 2021 and 2100 at a rate of more than 1% per year (Bhattacharjee et al., 2024).

The World Health Organization reports a high percentage of infertility cases in men, characterized by decreased sperm quality in terms of motility (sperm doesn't move forward, swims in circles, twitches, and is immotile), morphology (abnormal shapes in head, neck, and tail), and even DNA integrity (Saeidpour et al., 2024). Sperm is a crucial component in the distribution of genetic material during fertilization (Turner et al., 2020). Low sperm concentration, non-progressive motility, and morphological abnormalities can reduce the sperm's ability to actively navigate the female reproductive tract, reach the oocyte, and fertilize it (Kumar & Singh, 2015; Montoto et al., 2011).

Environmental factors can modulate reproductive problems experienced by men. In line with this, research conducted by Mai et al. (2023) found that environmental and job factors are associated with changes in male sperm quality. Men whose jobs involve polluted environments are at risk of exposure to industrial pollutants with high levels of toxicity (Bocu et al., 2024). Sperm quality can be

detrimentally affected by this, one of which is exposure to heavy metals (Abilash & Sridharan, 2024). Heavy metals bioaccumulate in the environment, then enter the soil and drinking water supply systems through industrial and domestic waste discharges (Chao et al., 2023). Lead pollutants released in high amounts into water, soil, and air have adverse effects on humans (Raj & Das, 2023).

Haryanto (2016) in his research found that the average blood lead levels in Indonesia almost doubled in 2015 (>39.3 µg/dL) compared to 2011 (27.9 µg/dL). This finding exceeds the recommended blood lead level limit set by the WHO, which is 5.0 µg/dL. Lead exposure can induce oxidative stress conditions that will later affect male spermatozoa production (Chao et al., 2023). Excessive lead exposure can markedly impair semen parameters, including reductions in semen volume, sperm concentration, and total motility (Giulioni et al., 2023).

Lead toxicity on testicular function was reported by Mustafa (2023), who found a significant reduction in the total number of germ cells and an increase in Leydig interstitial cell abnormalities in the lead-induced animal group. In line with this, Rana et al. (2021) discovered in their study that SAC from aged garlic given to rats was able to significantly elevated testosterone levels in Leydig cells. Bani et al. (2024) proved the significant effect of BG extract administration on increasing Follicle Stimulating Hormone (FSH) levels and increased concentration and motility. Furthermore, research conducted by Amida et al. (2021) also showed improvements in the concentration, viability, and motility of rat sperm through administration of BG extract.

This research innovation was conducted using whey kefir that contains yeast, lactic acid bacteria, and acetic acid bacteria for fermentation process. Fermentation is known to increase nutrient bioavailability, modify sensory properties, and produce bioactive components from SBG (Zeng et al., 2023). The increased concentration of bioactive compounds includes S-allylcysteine and flavonoid (Libero et al., 2024). S-allyl cysteine (SAC) is a unique organosulfur compound found in SBG and BG with promising positive effects for improving body health by fighting free radicals (Yeni et al., 2024). Therefore, solo black garlic fermented with whey kefir has the potential to increase concentration and motility, as well as reduce morphological abnormalities of spermatozoa in mice induced by lead acetate. This study aims to assess the effect of solo black garlic (SBG) on spermatozoa quality in mice induced to lead acetate.

METHOD

Preparation of *Solo Black Garlic* extract stock

This research was conducted at the Animal Reproduction and Development Laboratory of Sunan Gunung Djati State Islamic University, Bandung. In the initial stage, fresh single garlic (BPT) was fermented in whey kefir medium using the soaking method for 7 days. The following process was heating (aging) in an oven at a temperature of 60-70ºC and humidity of 70-80% for 21 days. The SBG extract was then prepared according to the methods described by Ghafil & Al-Taee (2020) and Ghalehkandi (2014).

Chemical testing of Solo Black Garlic

The pH test was performed according to the methodology of Ai & Huong (2018) by weighing 1 gram of crushed onion sample, dissolving it in 100 ml of distilled water, and thereafter measuring the pH level with a pH meter. The total flavonoid test was based on the studies of Afiati et al. (2020), Saputri (2019), and Aprianti (2023). The total flavonoid content was measured using a UV-Vis spectrophotometer at a wavelength of 428 nm. Furthermore, the total phenolic content test was carried out using the *Folin-Ciocalteu* method, referring to Solichah & Herdyastuti (2021) and Thalia et al. (2020), using UV-Vis spectrophotometry at a wavelength of 766 nm.

Animal preparations

Twenty-five Swiss Webster male mice (*Mus musculus*), aged 12-14 weeks and weighing

20-35 grams, were acclimated for 7 days. The mice were housed in ventilated plastic cages measuring 26 x 20 x 19 cm with wood husk bedding, maintained in a 12-hour light/dark cycle with a constant room temperature of 23 ± 2°C and a relative humidity of 50 ± 10% (Zi et al., 2022). All animals were provided with standard pellets and drinking water *ad libitum*. After acclimatization, mice that met the criteria were randomly assigned to five treatments (n = 5 per group). The sample size in this study was calculated following the Federer formula ((T-1) (N-1) > 15)). The simple random sampling method was carried out by labelling and randomly drawing all test animals using the Randomizer website (https://www.randomizer.org/).

Experimental design

The treatment consisted of five groups, namely: KN (negative control only given distilled water), KP (positive control only given lead acetate dose of 75 mg/kg BW), SBG-60 (lead acetate dose of 75 mg/kg BW and SBG dose of 60 mg/kg BW), SBG-120 (lead acetate dose of 75 mg/kg BW and SBG dose of 120 mg/kg BW), and SBG-180 (lead acetate dose of 75 mg/kg BW and SBG dose of 180 mg/kg BW). All substances were administered orally for a period of 30 days. Substance induction was carried out every day with a 2-hour gap between SBG extract and lead acetate to maximize absorption and prevent interactions between substances in the body (Suru, 2008). The body weight of mice in all treatments was measured every 3 days.

Measuring mouse sperm quality

On day 31, all mice were sacrificed by cervical dislocation and then dissected to obtain the cauda epididymis. Procedures of euthanasia are conducted based on the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2020 Edition (Leary et al., 2020). Surgical and sperm extraction procedures was carried out following the protocol from Martinez (2022). Then, the cauda epididymis was placed on a watch glass containing 1 mL of 0.9% NaCl to be minced, thereby suspending the sperm fluid. The resulting suspension was then observed for concentration, motility, and morphological abnormalities using a light microscope.

Determination of sperm concentration

The concentration of the spermatozoa suspension was observed using a Neubauer counting chamber consist of 9 large squares of 1mm each with 25 squares inside of the 16 mini-squares 0.05 × 0.05 mm each, and 0.1mm deep. The observation procedure was based on the method from Rombe et al. (2023). The sperm count was calculated using the following formula, where N is the average number of spermatozoa counted from the entire chamber (Ihsani et al., 2019). The dilution factor used is 100 times.

$$Concentration = N \times 5 \times 10^4 \times dilution\ factor$$

Examples: N = total 7.8 spermatozoa from 5 squares
Sperm concentration $= 7.8 \times 5 \times 10^4 \times 100$
$= 39.000.000$
$= 39 \times 10^6$/ml

Determination of sperm motility

Motility was assessed by observing the movement patterns of 200 spermatozoa per replicate. The magnification of the light microscope is set at 100x. Observations were conducted at room temperature (25-26°C) with a maximum duration of 60 minutes after collection and dilution (Agarwal et al., 2021). Each sperm cell was categorized as progressively motile, non-progressively motile, or immotile. The percentage of spermatozoa motility was then determined using the following formula (Irvanto et al., 2021).

$$(\%) = \frac{Progressive\ spermatozoa\ count}{Total\ spermatozoa\ counted} \times 100\%$$

Determination of sperm abnormality

Sperm abnormalities were observed by preparing slides from the samples, staining them with eosin-nigrosin and then observing them under a light microscope in 100x magnification. Total of 200 cells per replicate were observed.

Abnormality refers to morphological discrepancies in the head (large heads, hookless, amorphous, and irregular shapes), neck (bent necks, kinks, and abnormal neck attachments), and tail (broken, distal bent, hairpin loops, coiled, and tailless) of the sperm (Mathur et al., 2023). The percentage of normal and abnormal cells was then calculated using the following formula (Sitepu et al., 2023).

$$(\%) = \frac{Number\ of\ abnormal\ spermatozoa}{Total\ spermatozoa\ counted} \times 100\%$$

Statistical analysis

Data were analyzed using the Shapiro-Wilk test and Levene's test. A one-way analysis of variance (ANOVA) was performed if $p > 0.05$ and a Kruskal-Wallis test if $p < 0.05$. Then, testing was continued with the Duncan post hoc test or the post hoc nonparametric independent samples test. Statistical analysis was performed using SPSS software version 25.0, with $p < 0.05$ considered statistically significant and 95% Confidence Interval.

Ethical approval

All activities involving animals in this study was approved by the the Research Ethics Committee of the University of Padjadjaran, Bandung, with number 539/UN6.KEP/EC/2025. Any potential animals suffering is minimized by adhering to the 3R principle (replacement, reduction, refinement) and the 5F principle (five freedoms).

RESULTS AND DISCUSSION

Chemical properties of Solo Black Garlic

The results of chemical tests on pH values, flavonoid and phenolic content are presented in Table 1.

Table 1. Value of pH, flavonoid, and polyphenol content of single garlic and SBG

Parameter	Single Garlic	Solo Black Garlic
pH (± SD)	6,60 ± 0,06	4,2 ± 0,06
Flavonoid content (mgQE/g) ± SD)	13,10 ± 0,007	67,76 ± 0,047
Polyphenol content (mgGAE/g) ± SD)	73,37 ± 0,141	236,37 ± 0,374

Table 1 shows that SBG has a lower pH (4.2) than fresh single garlic (6.6) due to its higher acidity after aging. The decrease in pH in SBG occurs due to the Maillard reaction during aging (Ai & Huong, 2018). During the soaking process, molecules are transferred by osmosis. A semipermeable membrane with a high concentration allows the low-concentration whey kefir solution to flow toward the membrane in the single garlic clove, lowering the pH (Yahya, 2015). Additionally, the pH value is also closely related to phenolic content and structural changes in phytochemicals during fermentation (Hur et al., 2014).

The Table 1 indicates that as black garlic ages, its total flavonoid content increases. According to Choi et al. (2014), the aging process in solo black garlic increases the free fraction of phenolic acids, resulting in an increase in free phenol forms. Increased acidity during fermentation also contributes to this high flavonoid value by facilitating the release of flavonoid components (Setiyoningrum et al., 2022). Flavonoids reduce free radicals by three different mechanisms: delaying the production of reactive oxygen species (ROS), degrading ROS, and regulating/protecting with antioxidants (Susila Ningsih et al., 2023).

Fermentation treatment causes a significant decrease in pH due to lactic acid bacteria and acetic acid bacteria. According to Lestari (2017), the influence of pH is a significant factor on organic compounds, including phenol. Under alkaline conditions, the decrease in concentration will be greater because it will affect the photocatalytic process on the adsorbed dye. Consequently, the phenolic concentration will be much more stable when the pH tends to be acidic. Because phenolic substances include hydroxyl groups, they can contribute hydrogen and counteract free radicals' electron deficiency (Andriani & Murtisiwi, 2018).

Mice body weight during treatment

The differences of the body weight and weight gain of mice in all treatment groups in

Figure 1 shows statistically significant differences ($p < 0.05$).

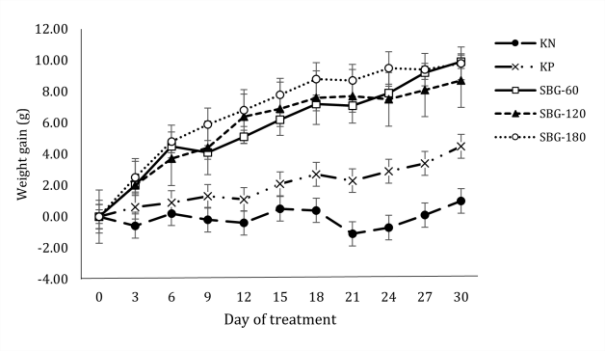

Figure 1. The weight change of mice across all groups over 30 days treatment

A drastic increase in weight was observed from day 3 to day 30 in the SBG treatment groups (SBG-60, SBG-120, and SBG-180), with weight differences reaching 9.90, 8.70, and 9.80 grams, respectively. In the control groups (KN and KP), weight changes did not exhibit drastic increases and tended to remain stable.

The correlation between weight gain and SBG administration may stem from the bioactive compounds contained within. SBG compounds can act as a supplement that promotes weight gain by increasing the rate of feed consumption in mice. Some compounds with high concentrations in SBG include S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC), and melanoidin (Amor et al., 2019). The high nutritional content and benefits of SBG support its potential to regulate metabolic rate and immunity. Such actions can lead to improved growth performance due to the innate immune response provided by SBG (Nguyen et al., 2025).

Mice sperm concentration

Data obtained from measuring the sperm concentration of male mice across all treatment groups indicated a significant effect ($p < 0.05$) due to lead acetate and SBG administration (Figure 2).

The KP group had the lowest average sperm concentration (15.00×10^6/ml), and the KN group had the highest (37.33×10^6/ml). This indicates that lead significantly reduced sperm concentration in the KP group

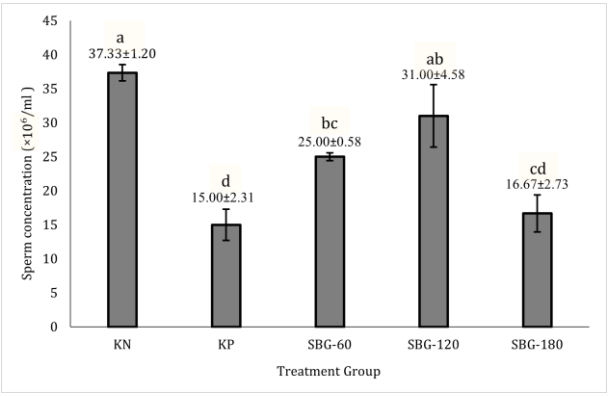

Figure 2. Average percentage of sperm concentration (KN: negative control, KP: positive control, SBG-60: SBG 60 mg/kg BW, SBG-120: SBG 120 mg/kg B1, SBG-180: SBG 180 mg/kg BW). Data are presented as mean ± SE from five biological replicates, each analyzed in triplicate technical replicates. Different alphabet (a, b, c) indicated significant differences among treatment groups ($p < 0.05$).

In the SBG treatment, the SBG-120 group (31.00×10^6/ml) with lead acetate induction and SBG supplementation showed no significant difference ($p > 0.05$) from the KN group (37.33×10^6/ml). Furthermore, the SBG-180 (16.67×10^6/ml) and SBG-60 (31.00×10^6/ml) groups showed no significant differences ($p > 0.05$).

The normal sperm concentration is > 20 million/ml of semen, and < 20 million/ml is considered infertile (Fauziyah & Dwijananti, 2013). In terms of lead exposure mechanisms, the testes are the most sensitive organs due to their intense cellular activity (Massányi et al., 2020). Lead exposure to the KP group was suspected to ROS production, which triggers lipid peroxidation through diffusion across the sperm membrane, which contains high levels of polyunsaturated fatty acids (Asadpour et al., 2013). Testicular function can also suffer primary damage due to impaired germ cell integrity, resulting in decreased sperm count (Ramos-Treviño et al., 2018). In this study, SBG administration demonstrated its ability to maintain and reverse the negative effects of lead

acetate administration on sperm production at high concentrations, particularly in the SBG-120 group. This study aligns with research by Bani et al. (2024), who found that administering black garlic (BG) to mice exposed to MSG increased sperm concentration. The flavonoid activity of BG is known to stabilize free radicals and inhibit lipid peroxidation. Furthermore, administering garlic to mice exposed to lead has been shown to reduce lead accumulation and oxidative stress (Nasr et al., 2017).

The SBG dose of 60 mg/kg BW was deemed insufficient to balance the high oxidant levels caused by lead. This dose may contain fewer antioxidants, which prevents it from restoring the balance of oxidation and reduction, making it insufficient for maintaining reproductive cell metabolism and producing sperm concentrations (Kowalczyk, 2021). The highest dose of SBG (180 mg/kg BW) decreased sperm concentration suspected to be due to the excessively high antioxidant content, preventing redox homeostasis (Panner Selvam et al., 2020).

Mice sperm motility

Regarding sperm motility, this study showed a significant difference ($p < 0.05$). The KP group had the lowest motility percentage, at only 5.83%. The KN group had the highest motility score at 81.33%. In preventing the adverse effects of lead, the treatment group that achieved the most optimal results was the one given SBG-120, with a motility percentage of 80.83%. The SBG-180 group had a motility percentage of 56.83%, and the SBG-60 group had a motility percentage of 18.83% (Figure 3).

Only the KN and SBG-120 groups showed no significant differences in motility ($p > 0.05$). Sperm with a good motility percentage are those in which >50% had progressive motility (Fauziyah & Dwijananti, 2013). Low motility in KP group is caused by lead induction that believed can modulates the formation of lipid peroxidation from ROS and damages sperm mitochondrial DNA, which is essential for motility (Behnejad et al., 2025)

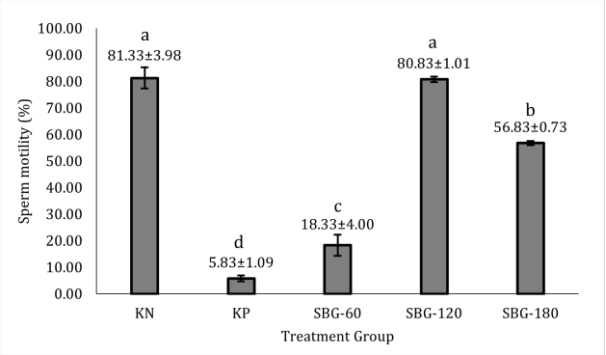

Figure 3. Average percentage of sperm motility (KN: negative control, KP: positive control, SBG-60: SBG 60 mg/kg BW, SBG-120: SBG 120 mg/kg B1, SBG-180: SBG 180 mg/kg BW). Data are presented as mean ± SE from five biological replicates (five individual mice per group), each analyzed in triplicate technical replicates. Different alphabet (a, b, c) indicated significant differences among treatment groups ($p < 0.05$).

In turn, this results in the loss of as much as 60% of sperm polyunsaturated fatty acids, impaired sperm membrane permeability, and inhibited flagellar motility due to reduced sperm ATP (Bani et al., 2024). The SAC compound in SBG is known to prevent this decline in total sperm motility by enhancing the testes response to oxidative stress (Takemura et al., 2014).

Mice sperm abnormality

The data obtained on the percentage of spermatozoa abnormalities showed a significant difference ($p < 0.05$). The KP group, which was treated only with lead, showed the highest increase in spermatozoa abnormalities, reaching 86%. In contrast to the KP group, the KN group showed the lowest abnormality rate, at 27.17%. In the group treated with SBG and lead, SBG-120 had the lowest abnormality rate at 30.50%, compared to SBG-60 at 41.67% and SBG-180 at 44.33% (Figure 4).

The WHO lower limit for normal spermatozoa morphology is ≥ 4% (Agarwal et al., 2021). In KP mice group exposed to lead, disruptions in sperm head and neck differentiation can occur because lead stimulates the abnormal expression of vital genes in spermatogenesis (Xie et al., 2020). The

variety of sperm abnormality forms found in this study is presented in Figure 5.

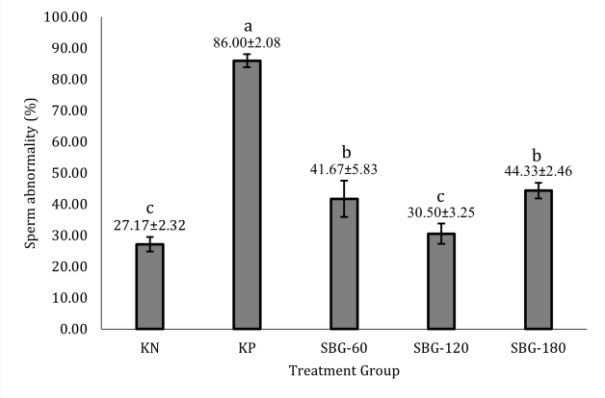

Figure 4. Average percentage of sperm abnormality (KN: negative control, KP: positive control, SBG-60: SBG 60 mg/kg BW, SBG-120: SBG 120 mg/kg B1, SBG-180: SBG 180 mg/kg BW). Data are presented as mean ± SE from five biological replicates (five individual mice per group), each analyzed in triplicate technical replicates. Different alphabet (a, b, c) indicated significant differences among treatment groups ($p < 0.05$).

In this study, the SBG-120 group exhibited abnormal values that were not significantly different ($p > 0.05$) from those of the KN group. Therefore, SBG at a dose of 120 mg/kg BW provided the best preventive effect compared to the doses of SBG-60 and SBG-180. SAC is known to increase endogenous antioxidant levels and donate protons to ROS to restore cellular homeostasis following oxidative stress (Becerril-Chávez et al., 2017). This results in suppression of the adverse effects of ROS interference on spermatozoa differentiation.

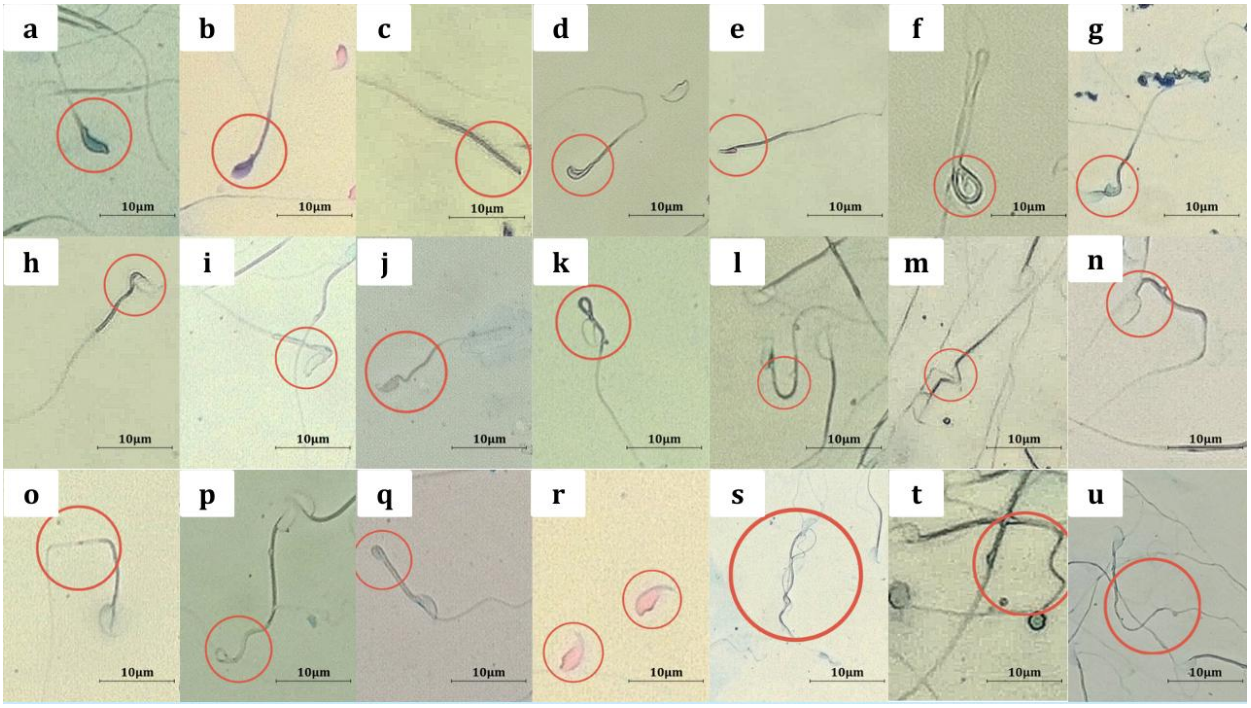

Figure 5. Variations in the shape of spermatozoa abnormalities. (a-g) Abnormalities of the spermatozoon head, (h-o) abnormalities of the spermatozoon neck and midpiece, (p-u) abnormalities of the spermatozoon tail

Optimal dose of SBG in improving sperm quality in mice

In this study, administration of lead acetate at a dose of 75 mg/kg BW caused significant degradation of spermatozoa quality. The KN group in this study had the best sperm quality values across all parameters. This reflects the stable and normal reproductive condition of the KN group throughout the treatment period.

The SBG-120 group showed the best results after KN, with increased concentration, motility, and reduced sperm abnormalities. The effectiveness of SBG in preventing the adverse

effects of lead is based on compounds produced through the fermentation process with kefir whey and aging at controlled temperature and humidity for 21 days. This process activates various reactions that lead to an increase in the bioactive compounds in onions.

The kefir whey used in the fermentation process contains various microbes, including *Saccharomyces cerevisiae*, which has been shown to enhance the antioxidant capacity of SBG. This high antioxidant capacity correlates with increased levels of flavonoids and polyphenols in SBG during aging (Setiyoningrum et al., 2022). Other compounds that increase with aging include allicin, SAC, Diallyl Disulfide (DADS), Diallyl Sulfide (DAS), and melanoidins, which are readily absorbed in the intestine (Afzaal et al., 2021). These organosulfur compounds act as oxidants that readily penetrate phospholipid membranes, thereby reducing lead levels in the blood and tissues. Furthermore, these compounds can increase lead concentrations in urine and feces (Senapati et al., 2001; Sharma et al., 2010). Various compounds in SBG provide spermatoprotective effects against the harmful impacts of toxic lead acetate compounds.

The abundance of compounds in SBG and its potential for spermatozoa recovery are known to be dose-limited. This study found that SBG at a dose of 120 mg/kg BW had the most significant effect on improving all three spermatozoa quality parameters compared to doses of 60 mg/kg BW and 180 mg/kg BW. This finding aligns with the results obtained by Omotoso et al. (2009), which indicate that a dose of garlic extract that is too low or too high poses a risk of disrupting antioxidant function, as testicular cells are susceptible to oxidative stress from chemicals.

CONCLUSION

The results of this study showed a significant effect of SBG fermented with whey kefir on spermatozoa quality in male mice induced by lead acetate. SBG at 120 mg/kg BW was the optimum dose at mitigated lead-induced declines in spermatozoa quality in mice as indicated by the spermatozoa concentration (31.00×10^6/ml), motility (80.83%), and abnormalities (30.50%), which were not statistically significantly different ($p < 0.05$) from negative control group (KN).

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