

## THE EFFECT OF LEMON (*CITRUS LIMON*) EXTRACTS ON THE QUANTITY AND QUALITY OF MICE (*MUS MUSCULUS*) SPERM

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**Abstract:** Vitamin C has been proved as a nutrient to improve the quality of sperm. Society believed that consuming the lemons could potentially enhance the sperm quality of humans. However, the appropriate concentration should be well studied to obtain the optimum concentration to improve the sperm quality and quantity. The present research tried to provide information on how lemon could improve the sperm quality by designing a true experimental using a series concentration of lemon extract (25%, 50% and 75 % concentrations) given to the male mice (*Mus musculus*). The investigation was made by giving the lemon treatment three times a day for 5 weeks. To investigate the effect of lemon extract, the mice sperm were taken from the epididymis and observed using a multimedia microscope and counted using Neubauer's counting rooms, while motility and morphology were observed using object-glass. The result showed that the high concentration of lemon could not provide the greatest improvement of sperm quality and quantity. The optimum condition was seen in 25% of lemon extract, where the increase of lemon concentration suppressed the lemon improvement effect, which reduced the sperm quality and quantity. However, the improvement was still made if the result was compared to control, meaning consuming lemon was better than consuming any lemon treatment. The result was in accordance with quality improvement of sperm, where 25% of lemon concentration treatment provided the highest average motility and normal morphology of sperm. However, the high concentration of lemon extract (50% and 75% of lemon concentrations) provided a lower effect due to the adverse effect. The result proved that lemon could be used to boost the quality and quantity of sperm in an appropriate concentration where the excess lemon extract could reduce the effect of lemon in improving sperm quality and quantity.

**Keywords:** Lemon; Sperm; Vitamin C; *Mus musculus*

**Abstrak:** Vitamin C telah terbukti sebagai nutrisi untuk meningkatkan kualitas sperma dimana masyarakat percaya bahwa mengkonsumsi lemon berpotensi meningkatkan kualitas sperma manusia. Namun, konsentrasi yang tepat harus dipelajari dengan baik untuk mendapatkan konsentrasi yang optimal untuk meningkatkan kualitas dan kuantitas sperma. Penelitian ini mencoba memberikan informasi bagaimana lemon dapat meningkatkan kualitas sperma dengan merancang eksperimen nyata menggunakan serangkaian konsentrasi ekstrak lemon (konsentrasi 25%, 50% dan 75%) yang diberikan kepada mencit jantan (*Mus musculus*). Penyelidikan dilakukan dengan memberikan ekstrak lemon sebanyak tiga kali dalam sehari selama 5 minggu. Untuk mengetahui pengaruh ekstrak lemon, sperma mencit diambil dari epididimis dan

diamati menggunakan mikroskop multimedia dan dihitung menggunakan kamar hitung Neubauer, sedangkan motilitas dan morfologi diamati menggunakan kaca objek. Hasil penelitian menunjukkan bahwa konsentrasi lemon yang tinggi tidak dapat memberikan peningkatan kualitas dan kuantitas sperma yang tertinggi. Kondisi optimum terlihat pada ekstrak lemon 25% dimana peningkatan konsentrasi lemon menekan efek perbaikan lemon yang menurunkan kualitas dan kuantitas sperma. Namun perbaikan tetap dilakukan jika hasilnya dibandingkan dengan kontrol, artinya mengkonsumsi lemon lebih baik daripada tanpa mengkonsumsi lemon. Hasil tersebut sesuai dengan peningkatan kualitas sperma dimana perlakuan konsentrasi lemon 25% memberikan rata-rata motilitas dan morfologi sperma yang normal paling tinggi. Namun, konsentrasi tinggi ekstrak lemon (50% dan 75% konsentrasi lemon) memberikan efek yang lebih rendah karena efek samping. Hasil penelitian membuktikan bahwa lemon dapat digunakan untuk meningkatkan kualitas dan kuantitas sperma dalam konsentrasi yang sesuai dimana kelebihan ekstrak lemon dapat mengurangi efek lemon dalam meningkatkan kualitas dan kuantitas sperma.

**Kata kunci:** Lemon; Sperma; Vitamin C; *Mus musculus*

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## Introduction

Fertility and infertility are conditions related to producing offspring in a partner. Around the world, approximately 10 % population has infertility, and about 8-12 % are reproductive-age couples (Ombelet et al., 2008; Direkvand-Moghadam et al., 2014). Meanwhile, 15-20% of all partners are infertile (Indonesian Department of Health, 2013). Male or female can be a factor causing infertility, and most previous studies stated 26-30 % of fertility was coming from male organisms (Vitrikas and Lindsay, 2015). The cause of male infertility was the quality and quantity of sperm that failed to fertilize the ovum even though the couple has had intercourse without using protection for at least one year (Zegers-Hochschild, 2009). World Health Organization (WHO) determines that a normal sperm consists of at least 15 million per mL of ejaculate for concentration, 32% motility (movement) of sperm indicates a progressive condition (advanced and fast) and has 14 % normal morphology (shape) (World Health Organization, 2010). The condition where the sperm has quality below normal standards or are known as abnormal. Various research has shown that abnormalities of sperm, especially concentration and motility, are the main causes of male infertility (Miyamoto, 2012; Abarikwu, 2013; Sharma, 2017; Wdowiak, 2019; Babakhanzadeh, 2020).

Several factors that affect the quality of sperm include radiation (Gorpinchenko, 2014; Kesari, 2018), nutrition (Dewantari, 2013; Martinez-Soto, 2016; Salas-Huetos, 2018), lifestyle (Durairajanayagam, 2018; Ilacqua, 2018), age (Harris, 2011; Silea, 2019), psychological (Collodola, 2008; Janevic, 2014) and

deletions occurred in the Y chromosome (Hanizar, 2004; Poongothai et al., 2009; Bansal, 2016; Colaco & Modi, 2018). The nutrients containing vitamin C and E could use to improve the quality of sperm [Ahmadi, 2016; Sutanto, 2017; Majzoub & Agarwal, 2018). To be more specific, the study about vitamin C proved that the men who consume 1000 milligrams of vitamin C per day experience an increase in the concentration and motility of sperm (Rafiee, 2016). The German Nutrition Society announced the adequacy of vitamin C, which was approximately 65 -90 mg /day for adolescents and 95 -110 mg/day for adults. The dosage requirements for males are higher than for females (German Nutrition Society, 2015). In animals, vitamin C has been shown to increase sperm count and motility of rats after being given the stress of swimming (Vijayprasaad, 2014) or exposed the cigarette smoke (Claudia, 2013).

One type of fruit that predominantly contains vitamin C is lemon (*Citrus limon*). The lemon extract contains several vitamins such as vitamin C (53 mg / L), vitamin A, vitamin B1, vitamin B2, vitamin B3, phenolic acids and flavonoids (Klimek-Szczykutowicz, 2020). Approximately 100 mL of lemon contains 50 mg Vitamin C, 8600 mg Potassium, 8452 mg Calcium and 6656 mg Phosphor (Chuku & Akani, 2015). In addition, the lemon pulp also contains Vitamin C of 130 mg, Vitamin A of 300 IU, mineral Phosphor of 22%, and less than 0.5% of other minerals (Chuku & Akani, 2015). The content of vitamins C in lemon is higher than lime (*Citrus aurantiifolia*). The lemon contains 48 mg of vitamin C, while lime contains only 38 mg of vitamin C per 100 mg of fresh flesh (Simona, 2011). The other researcher supported the previous report where there was 53,47 mg vitamin C in lemon and 35,67 mg in lime per 100 mL of flesh (Nangbes, 2014; Shaha & Paul, 2014). The high concentration of essential vitamins and minerals is potentially used in sperm development.

The present research aims to see how the lemon extract could support sperm development. The study would focus on the investigation of lemon extract treatment in the development of quality and quantity of mice (*Mus musculus*) sperm. The different concentrations of lemon extract will be used to see the optimal condition of treatment, which could give the best quantity and quality of mice sperm, including the sperm concentration, motility, and normal shape of sperm. The mechanism of lemon extract in support of sperm development will be discussed. The present study result could become important information, especially to treat patients who have fertility problems using a natural component in the lemon fruit.

## **Materials and Method**

### **Preparation of lemon juice extract**

The present research was experimental research using a specific lemon extract as the treatment to see how lemon extract affected sperm quality. 100 gram of local fresh lemon was cut in the middle and sliced into several pieces.

The lemon slices were squeezed using an orange juicer to extract the flesh. The extract lemon was determined as 100% of lemon extract. This study used a series of lemon extract concentrations to investigate the lemon effect's optimum condition in sperm development. The concentrations (v/v) were 25% (T1), 50% (T2), and 75% (T3). The dilution was conducted in the volumetric flask by adding a specific amount of distilled water into the lemon extract until the desired concentration was achieved (Penniston, K. L. et al., 2009).

### **Study the effect of the lemon extract on sperm quality and quantity**

The experimental animals used in this study were male mice (*Mus musculus*) with a specific age of 4 weeks with a weight of  $\pm$  10-11g. All the sample used was obtained from the laboratory of animal physiology, Islamic University of Malang. All the experimental research has been reviewed for ethical clearance by the animal care and use committee of Universitas Brawijaya, No: 1061-KEP-UB.

The study effect of lemon juice extract in the quality and quantity of male mice sperm began by dividing the group into four groups of treatment based on the variety of concentrations of lemon juice extract (v/v): 25% (T1), 50% (T2), 75% (T3) and a control (without treating with lemon juice). Before being treated with the lemon extract, the mice were acclimatized for 7 days to ensure all the samples were treated with the same condition. After acclimatization, the treatment was conducted by orally giving the sample a specific concentration of lemon using a dose of 0.5 mL of a feeding tube 3 times a day where the main food in the form of pellets was given regularly and provided with the distilled water by *ad libitum*. 6 replications would conduct each treatment to ensure the experimental was repeatable and treated for 5 weeks of the investigation period. The mice were kept in plastic cages with woven wire caps where each cage contained 5 animals.

### **Investigate the effect of the lemon extract on sperm quality and quantity**

The observation of mice sperm was conducted after the mice were 10 weeks old by killing the animals using the cervical dislocation method. The epididymis was taken, cut into small pieces using scissors, put into a petri dish containing 0.9% a sterile physiological NaCl and stirred with a glass stirrer to form a suspension of sperm. The sperm concentration was calculated by means of one drop of the homogeneous suspension placed on the Neubauer counting chamber and observed under a multimedia microscope (Olympus Bx43 with DP22 Camera) with a magnification of 400 times. The number of sperm concentrations was calculated using the equation below (Eq. 1) (Tan, E. et al. 2010).

$$\text{The sperm concentration } \left( \frac{\text{millions}}{\text{mL}} \right) = \text{The number of observed sperm} \times \text{dilution value} \dots \dots (1)$$

The motility of the sperm was determined by dropping one drop of sperm suspension on the object-glass, close with the glass cover, and observed under a specific 400-fold magnification microscope (Olympus Bx43 with DP22 Camera). The observation of sperm was done in 4 to 6 fields of view to obtain 100 sperm and observed. The observation was assessed using a specific criterion shown below.

- a (progressive motility = moving forward and fast);
- b (non-progressive motility = moving neither forward nor fast); and
- c (immotility = not moving).

To calculate the motility value, the calculation was made using equation 2 (Kumar & Singh, 2015):

$$\text{Motility value} = \frac{a + b}{a + b + c} \times 100\% \dots \dots \dots (2)$$

The morphology of sperm was observed on smear preparations by dropping one drop of sperm on a glass object and allowed to dry. The glass object was then soaked in absolute methanol (Merck) for 5 minutes, rinsed with distilled water and dried. Furthermore, the preparations were immersed in Safranin solution (Arkitos Chemical) for 5 minutes, immersed in Phosphate Buffer solution (Merck) 3 times, and immersed in Crystal Violet solution (Merck) for 5 minutes. In the final step, the sample of sperm was washed under running water, and after drying, the preparations were observed under a microscope with a magnification of 1000 times. The morphology of sperm was categorized as normal if the sperm had a tapered head and hooked ends, followed by a straight neck and a long, non-bent tail (Albrechtova, 2014). Conversely, the sperm was abnormal if the head appeared small, shapeless, or unlinked (Rasgele, 2014), while the tail was short, double, or curled. Some of the possible abnormal sperm necks included asymmetrical sperm heads, thicker and sharply bent (Safitri & Hanizar, 2019). The percentage of normal sperm was calculated using the equation below.

$$\text{The number of sperm} = \frac{\text{The number of observed normal sperm}}{\text{Total observed sperm}} \times 100\% \dots \dots \dots (3)$$

## Result

Figure 1 shows the effect of the lemon extract on sperm concentration. The results performed that the lemon extract influenced all parameters of sperm. The 25% lemon concentration (T1) produced approximately 8.9% of sperm concentration of 8.9 million sperm per 100 mL of sample. The result proved the treatment using 25% of lemon concentration became the highest mean sperm concentration compared to other concentration treatments while the control

showed the lowest results (2.09%) compared to other treatments ( $p < 0.05$ ). The Duncan's test (table 1) showed that 50% concentration (T2) and 75% concentration) (T3) of lemon extracts were not giving any significant difference where the improvement was only 0.55% by increasing the lemon extract from 50% to 75%. The result showed that the higher the concentration of lemon did not give any improvement in sperm concentration.

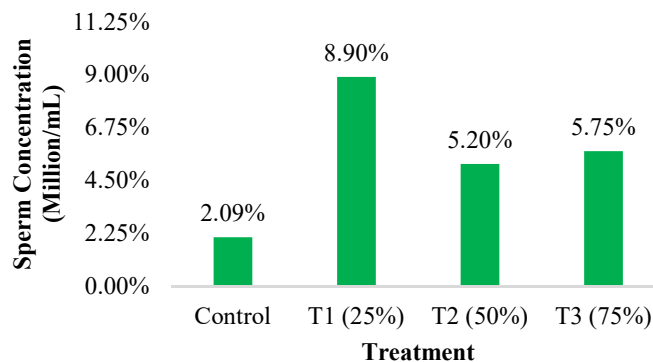


Figure 1. The average sperm concentration for any given treatment

In line with the observations of sperm concentration, the mean percentage of sperm motility showed that the treatment with the concentration of 25% lemon produced the highest mean percentage of motility compared to other treatments (figure 2) by showing approximately 20% higher in the number of sperm motility compared to control. The ANOVA test analysis proved by showing a significant difference between any treatments ( $p < 0.05$ ). However, Duncan's test confirmed that an increase did not follow the increase of lemon concentration in the mean sperm motility. The main reason was the difference between the treated samples and control was relatively small.

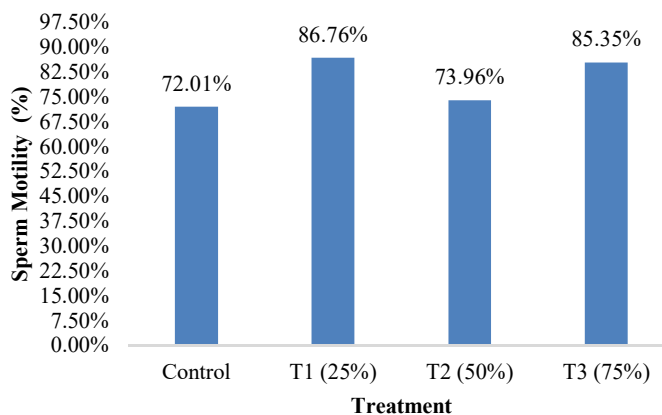


Figure 2. The average sperm motility for any given treatment

The study was continued by investigating the number of sperm with normal morphology. The result in figure 3 showed that the first treatment using 25% of lemon extract had the highest percentage of normal sperm morphology compared to other treatments by showing 11% higher than control. However, the trends showed a decrease in percentage by increasing the lemon extract concentration. Treating with 75% of lemon concentration formed abnormal sperm morphology indicated by the forms of the abnormal tail (curved and twisted) and the curled neck. The comparison between the normal and abnormal sperm is shown in figure 4.

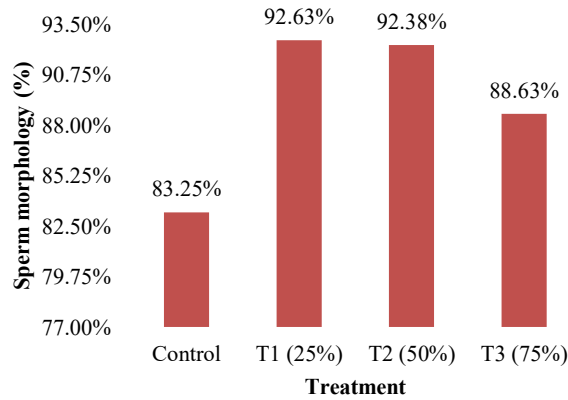


Figure 3. The average normal sperm morphology for any given treatment

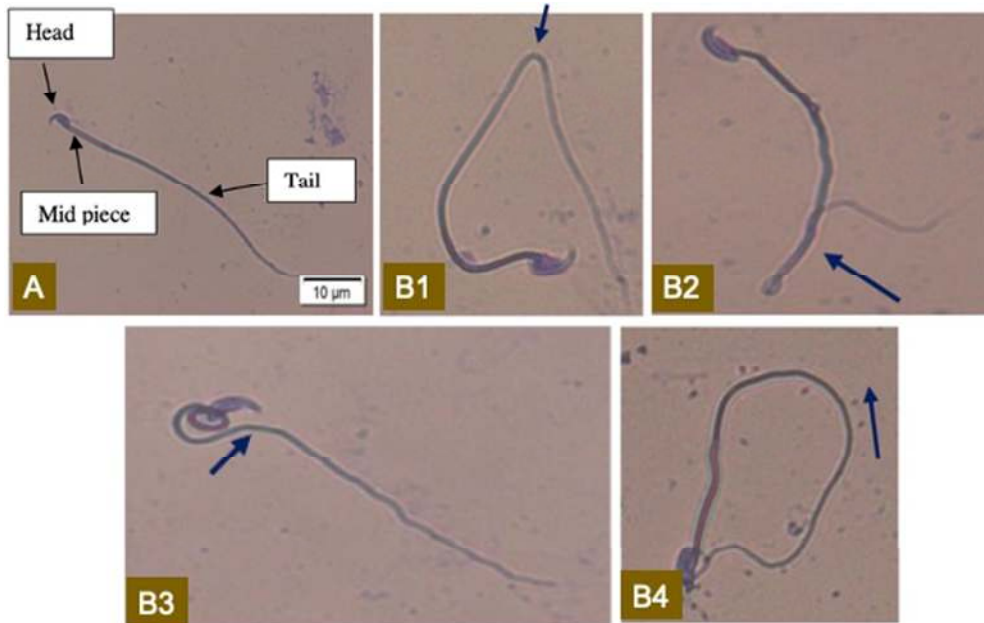


Figure 4. (A) the normal morphology of sperm, (B1 and B4) abnormally curved tail, (B2) twisted tail, and (B3) curled neck.

## Discussion

The study has shown that the lemon extract treatment could positively impact sperm development. In general, all the treatments showed higher concentration, motility, and normal morphology compared to control. Before explaining how the lemon extract could help develop sperm, we explain how sperm are produced.

The sperm production was placed in the seminiferous tubules of the testis and refined in the epididymis to become normal sperm capable of fertilizing the ovum. This process begins with the proliferation of spermatogonia, meiosis of the spermatocyte into spermatids, and the spermatids' change into functional sperm (Borg et al., 2010). Various external factors can be influenced during this process, such as nutrition, genetics, lifestyle, and environment (Blay et al. 2020). To be specific in nutrition, poor nutritional quality can inhibit puberty growth, thus impacting sterility. Spermatogenesis involves several hormones, and the most important one is the secretion hormone, which requires adequate nutrition to produce sperm (Ban & Zhao, 2018). The other hormones were Luteinizing Hormone (LH) which stimulates Leydig cells to secrete testosterone, and Follicle Stimulating Hormone (FSH), which stimulates Sertoli cells to release Androgen Binding Protein (ABP). These hormones work together to produce sperm ready to fertilize the ovum (Ramaswamy & Weinbauer, 2014).

The present research showed that the lemon could increase sperm concentration. The lemon contains vitamin C, which plays the role of an antioxidant or reducing agent in supporting the male reproductive system and maintaining sperm function, testicular structural integrity and increasing the work of hormones in stimulating testosterone production (Kapsul, 2011). Rahayu (2019) reported that vitamin C could boost the concentration, morphology, and testosterone serum (Rahayu et al., 2019). The high concentration of testosterone supported the conversion of spermatocytes to sperm and the maturation of sperm in the epididymis (Smith & Walker, 2014). As support, Ebesunun (2009) reported that Vitamin C could increase the number and motility of sperm (Ebesunun et al., 2009).

In general, all the treatment provides the improvement in sperm concentration by showing a higher number compared to control. The treatment produced the highest concentration of 25% of lemon extract, where lemon extract decreased sperm concentration. However, there was a slight increase of sperm concentration in 75% of lemon extract compared to 50% concentration, but the Duncan test analysis results showed that the difference was not significantly different. This indicates that increasing the dose has an adverse effect on the number of sperm where the proper concentration could potentially maximize the development of sperm. Nirmalasari (2017) supported the finding where the vitamin C contained in *Anadara granosa* L. could decrease the sperm concentration in the high concentration feeding (Nirmalasari, 2017). The excess



and lack of nutrition could have a negative effect on reproductive health, indicating the study of optimum concentration was important before doing treatment (Ferial & Muchlis, 2013; Sudarti et al., 2019).

The giving of lemon extract treatment also gives an increased response to the mobility of sperm. Al-sultani (2013) claimed that 0.06 mg / mL of Vitamin C on in-vitro activation of sperm could improve the percentage of motility and sperm quality (Al-sultani et al., 2013). The terms of quality were related to the “a” category determined by World Health Organization (WHO). The high-quality sperm was the one which showed the fast movement, forward or in a large circle, and category “b” as the non-progressive with a specific movement in a small circle, or only flagellum movements (World Health Organization, 2010). In this study, the motility of sperm in all the treatments showed a faster movement compared to control. The treatment using 25% of lemon extract provided the fastest motility compared to other conditions, showing that 25% of lemon extract provided high energy for sperm to do the activities.

The other possible reason for the high motility of sperm by treating with 25% of the lemon extract was the sperm morphology. The progressive quality of sperm motility occurs if the sperm have a normal shape. The morphology of normal sperm was shown by the perfect tapered head and hooked ends, followed by a straight neck and a long, non-bent tail. The sperm-like are observed for concentration and morphology and could move for motility observations (Cyrus et al., 2015). The result showed that the high motility in 25% of lemon extract treatment was supported by the high quantity of normal shape of sperm morphology. The number of normal sperm morphology decreased when the lemon extract concentration increased. The high concentration of lemon extract had a negative impact on the spermatozoa and affected the sperm morphology (Ferial & Muchlis, 2013; Nirmalasari, 2017).

The role of vitamin C in spermatozoa have been tested by treating the patient who underwent varicocele surgery. The patient was given two doses of 250 mg Vitamin C for 3 months. The result showed that there was an improvement in the patient sperm, especially the motility, morphology, viability, acrosome reaction, and DNA integrity (Cyrus et al., 2015; Fanaei et al., 2014; Arafa et al., 2020). The mechanism of vitamin C improved sperm quality, and quantity was begun during maturation in the epididymis. Vitamin C supports sperm development as additional energy that successfully ensures the spermatozoa process. The other role was an antioxidant, where the vitamin C in the lemon extract protected the sperm from free radicals that potentially disturb the spermatozoa process (Noorul et al., 2017). The free radicals, also known as Reactive Oxygen Species (ROS), were the substances that could inhibit sperm development because of their free ion radicals. The presence of vitamin C as the antioxidant stabilized the free ion radical to perform the neutral substances which effectively used in sperm development. However, the high amount of ROS

sometimes was higher than the number of antioxidants and initiated the cells to experience oxidative stress leading to cell damage (Nicol & Prasad, 2006). On the other hand, the high amount of antioxidants also negatively impacted where the exceeds of antioxidants could not be optimum to boost the sperm development, especially in the percentage of sperm motility.

The observations on sperm morphology also gave a response to an increase in the percentage of normal morphology by showing approximately 90% of normal morphology in the optimum condition. The pattern of improvement also shows the same trend where an increase did not follow the treatment of lemon concentration of 50% in the percentage of normal sperm. This finding indicated that the optimum concentration of lemon needed to obtain sperm with normal morphology was 25% of lemon extract. This vitamin works as a cleanser that has a broad spectrum against ROS, thereby successfully neutralizing the effects of DNA damage and ROS production (Greco et al., 2005; Tremellen, 2008; Wright et al., 2014).

In addition, the observations also showed the production of abnormal morphology. The sperm abnormality can occur in the seminiferous tubules, called the primary abnormalities, or at the stage of improvement in the epididymis, the secondary abnormality (Bertol et al., 2013). The abnormality was initiated by the lack of vitamin A containing the spermatogenesis process (Abdulkareema et al., 2004). The reduced concentration of vitamin A in rams resulted in various morphological abnormalities of sperm ranging from the shape of the giant, small, tapering, and pyriform heads, the midpiece and coiled tail. This abnormality is due to a reduction in the concentration of vitamin A at the epithelial stage of the head of the epididymis, which will reduce the synthesis of certain proteins such as transferrin and clustering, which were required for sperm maturation (Abdulkareema et al., 2004). However, no comprehensive data showed the level of vitamin A in each treatment of lemon extract given.

The lemon juice also contains several minerals such as potassium (K), Calcium (Ca), Phosphor (P), etc. Together with Na and Zn, the minerals were essential because they are components of various important enzymes. Therefore, suboptimal concentrations of these minerals are associated with male infertility. For example, Calcium could trigger acrosome reactions that support sperm motility (Harchegani et al., 2019; Halo et al., 2018). On the other hand, the potassium (K) concentration was related to testosterone production. At the same time, the amount of calcium content affected the increase of slow-moving and immotile sperm (Hamad et al., 2014).

## Conclusion

The role of lemon extract to support sperm development was complex. Each element, such as vitamin C, minerals, and the other vitamin contained in the lemon extract, could support the spermatozoa process. The present study proved

that the proper amount of lemon extract increased the quantity and quality of mice sperm by showing the improvement of concentration, motility, and normal morphology of mice sperm. The excess amount of lemon extract could not provide an additional function but show the opposite effect that could debilitate the function of lemon extract treatment.

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