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THE EFFECT OF LEMON (*CITRUS LIMON*) EXTRACT 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 and Lemon is well known as the source of Vitamin C. The present research aims to determine the effect of lemon extract on the quantity and quality of mice sperm. The research was a true experimental using a series concentration of lemon extract (25%, 50% and 75 % concentrations) given to the 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 observed using object glass. The result showed a significant effect of sperm quality and quantity where the sperm was health and form a normal shape. In terms of quantity, the optimum condition (25%) could boost the sperm concentration, motility, and morphology up to approximately 300%, 20%, and 10%, respectively. The high quality and quantity were because the lemon extract could become an antioxidant and nutrient which support the sperm development. However, the high concentration of lemon extract (50% and 75%) provided a lower effect due to the adverse effect meaning the information about the optimum concentration of lemon extract was mandatory before treating the mice for the further purpose.

Keywords: Lemon; Sperm; Vitamin C, Mice

Abstrak: Vitamin C telah terbukti menjadi nutrien yang dapat meningkatkan kualitas sperma dan Lemon adalah satu dari sekian banyak buah yang mengandung kaya vitamin C. Penelitian ini memiliki tujuan untuk mempelajari pengaruh pemberian ekstrak lemon terhadap kuantitas dan kualitas dari sperma tikus. Penelitian menggunakan desain *true experiment* melalui pemberian beberapa konsentrasi ekstrak lemon (25%, 50%, dan 75%) kepada sampel tikus selama 5 minggu. Untuk menguji pengaruh pemberian ekstrak lemon, sample sperma tikus diambil dari epididymis dan diobservasi menggunakan multimedia mikroskop dan dihitung menggunakan ruang hitung Neubauer. Angka Motality dan morfologi dari sperma selanjutnya diobservasi menggunakan gelas objek. Hasil penelitian menunjukkan pengaruh yang signifikan terhadap kualitas dan kuantitas sperma tikus dimana sperma tikus ditemukan sehat dan membentuk morfologi normal. Pemberian konsentrasi optimum memberikan peningkatan konsentrasi sperma, motility, dan pembentukan morfologi masing-masing sebesar 300%, 20%, dan 10%. Peningkatan kualitas dan kuantitas karena ekstrak lemon dapat menjadi antioksidan dan nutrient yang mendukung perkembangan sperma. Namun, pemberian konsentrasi yang tinggi menghasilkan efek yang lebih rendah dibandingkan kondisi optimum karena pengaruh yang berlawanan. Hal ini menunjukkan bahwa informasi tentang konsentrasi optimum

ekstrak lemon adalah keharusan sebelum memberikan perlakukan kepada tikus untuk keperluan lebih lanjut..

Kata kunci: Lemon; Sperma; Vitamin C, Mencit

Recommended APA Citation :
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Introduction

Fertility and infertility are conditions related to the ability to produce 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 in Indonesia, 15-20% of all partners are infertile (Indonesian Department of Health, 2013). Male or female can be a factor causing infertility, and most previous study stated 26-30 % of fertility was coming from male organism (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 consisted 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 have quality below normal standards or are known as abnormal. The 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 (Collodela, 2008; Janevic, 2014) and deletions occurred in the Y chromosome (Hanizar, 2004; Poongothai et al., 2009; Bansal, 2016; Colaco & Modi, 2018). The nutrients containing the 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 adequacy of vitamin C which was approximately 65 - 90 mg /day for adolescents and 95 -110 mg/day for adults, and the dosage requirements for male are higher than for female (German Nutrition Society, 2015). In animals, vitamin C has been shown to increase sperm count and motility of rat

after being given the stress of swimming (Vijayprasaad, 2014) or exposing the cigarette smoke (Claudia, 2013).

One type of fruit that predominantly contains vitamin C is lemon (Citrus limon). 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 potentially used in the sperm development.

The present research aims to see how the lemon extract could support the 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 concentration of lemon extract will be used to see the optimal condition of treatment which could potentially 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 the support of sperm development would be discussed. The present study result could become an important information specially to treat patient who have problem in fertility using a natural component in the lemon fruit.

Materials and Method

Preparation of lemon juice extract

The present research was an experimental research using a specific lemon extract as the treatment to see how lemon extract affected the 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. In this study, we used a series of concentration of lemon extract to investigate the optimum condition of lemon effect in sperm development. The concentrations (v/v) were 25% (T1), 50% (T2), 75% (T3). The dilution was conducted in the volumentric flask by adding a specific amount of distilled water into the lemon extract until the desired concentration achieved (Penniston, K. L. et al. 2009).

Study the effect of lemon extract in sperm quality and quantity

The experimental animals used in this study were male mice (*Mus musculus*) with a specific aged of 4 weeks with a weight of ± 10-11g. All the sample used was obtained from the laboratory of animal physiology, Islamic University of Malang. Before being treated with the lemon extract, the mice were acclimatized for 7 days to ensure all the sample treated with the same condition. All the experimental research has been reviewed for the ethical clearance by the animal care and use committee of Universitas Brawijaya, No: 1061-KEP-UB. \ The research began by doing the acclimatization for the mice for 7 days.

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 varies of concentration of lemon juice extract (v/v): 25% (T1), 50% (T2), 75% (T3) and a control (without treating with lemon juice). The lemon was orally given to the sample using a dose of 0.5 mL of feeding tube for 3 times a day where the main food in the form of pellets was given regularly and providing with the distilled water by ed libitum. Each treatment would be conducted by 6 replications to ensure the experimental was repeatable and treated for 5 weeks of investigation period. The mice were kept in plastic cages with woven wire caps where each cage contains 5 animals.

Investigate the effect of lemon extract in 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 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 concentration was calculated using the equation below (Eq. 1) (Tan, E. et al. 2010) .

$$The\ sperm\ concentration\ \left(\frac{millions}{mL}\right) = The\ number\ of\ obseved\ sperm\ x\ dilution\ value \quad (1)$$

from the observation was multiplied by the dilution value of the suspension to obtain the concentration in units of millions/mL.

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 of 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-progessive motility = moving neither forward nor fast); and
c (immotility = not moving).

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

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

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 were 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 head, thicker and sharply bent (Safitri & Hanizar, 2019). The percentage of normal sperm was calculated using the equation below.

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

Result

Figure 1 showed the effect of lemon extract on sperm concentration. In general, the results performed that the lemon extract influenced all parameters of sperm. The 25% lemon concentration (T1) produced the highest mean sperm concentration, while the control showed the lowest results compared to other treatments ($p < 0.05$). Furthermore, the Duncan's test (Table 1) showed that T2 (50% concentration) and T3 (75% concentration) were not giving any significant difference, meaning that the higher the concentration of lemon did not give any improvement in sperm concentration.

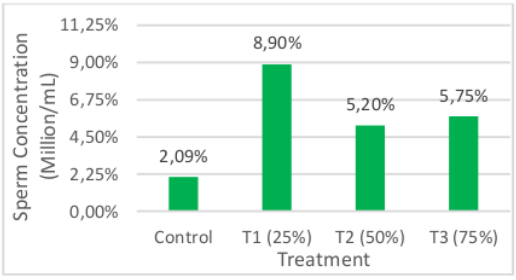

Figure 1. The average sperm concentration for any given treatment

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). The ANOVA test analysis proved by showing a significant difference between any treatments ($p < 0.05$). Meanwhile, Duncan's test confirmed that the increase of lemon concentration was not followed by an increase in the mean sperm motility.

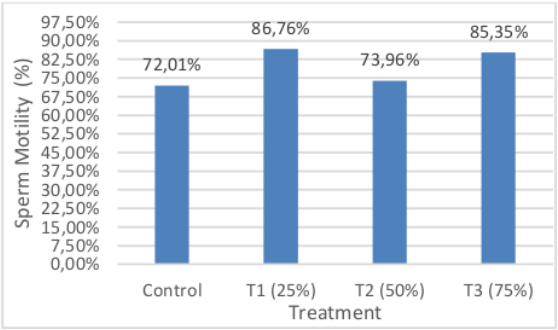

Figure 2. The average sperm motility for any given treatment

The study was continued by investigating the number of sperm which had a normal morphology. The result in Figure 3 showed that the first treatment (T1) had the highest percentage of normal sperm morphology compared to other treatments The trends showed a decreasing in percentage by increasing the concentration of lemon extract. However, there were small amount of abnormal sperm obtained in high concentration of lemon extract (T3) such as the forms of abnormal tail (curved and twisted), and the curled neck. The comparison between the normal and abnormal sperm was shown in Figure 4.

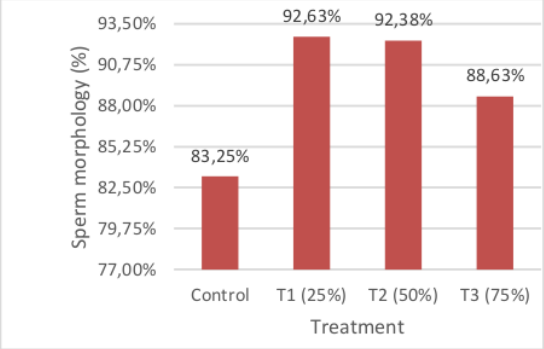

Figure 3. The average normal sperm morphology for any given treatment

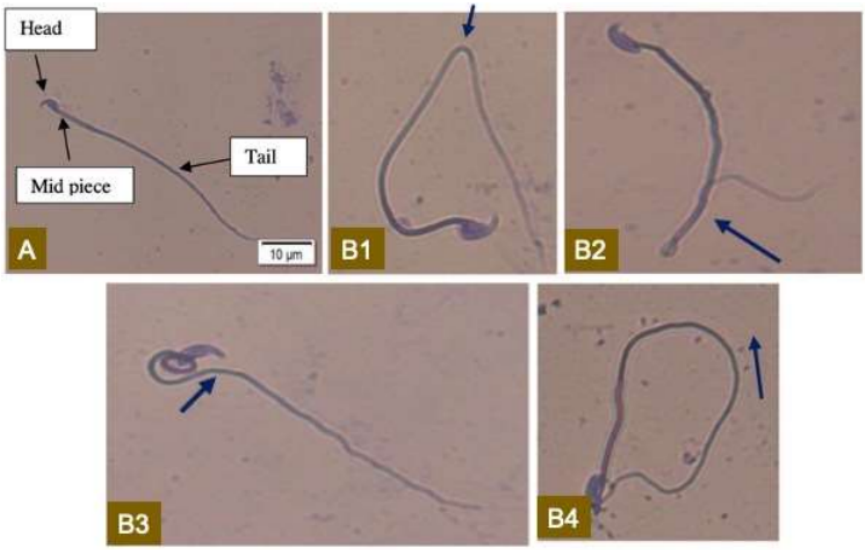

Figure 4. (A) the normal morphology of sperm, (B1 and B4) abnormal curved tail, (B2) twisted tail, and (B3) curled neck.

Discussion

The study have shown that the lemon extract treatment could provide a positive impact in the sperm development. In general, all the treatment showed the higher concentration, motility, and normal morphology compared to control. Before explaining how the lemon extract could help in developing of sperm, we would explain how sperm are produced.

The sperm production was placed in the seminferus 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 change of the spermatids into functional sperm (Borg et al., 2010). During this process, various external factors can be influenced such as nutrition, genetic factor, lifestyle, and environment (Blay, et al. 2020). To be specific in nutrition, the poor nutritional quality can lead to inhibition of puberty growth, thus impacting on sterility. The spermatogenesis involves several hormones and the most important one is the secretion hormone which required the adequate nutrition to produce sperm (Ban & Zhao, 2018). The other hormones were Luteinizing Hormone (LH) which stimulates Leidig cells to secrete testosterone and Follicle Stimulating Hormone (FHS) which stimulates Sertoli cells to release Andogen Binding Protein (ABP). These hormones work together to produce sperm that are ready to fertilize the ovum (Ramaswamy & Weinbauer, 2014).

The present research showed that the lemon can increase sperm concentration. The reason was the lemon contains vitamin C which played the role as 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 the 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 also the maturation of sperm in the epididymis (Smith & Walker, 2014). As support, Ebesunun (2009) reported that the 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 was 25% of lemon extract where the increase of lemon extract decreased sperm concentration. However, there was a slightly increase of sperm concentration in 75% of lemon extract compared to 50% concentration but the results of the Duncan test analysis showing that the different 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 containing *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 Vitamin C on invitro activation of sperm can improve the percentage of motility and sperm quality (Al-sultani et al., 2013). The terms of quality were related with the “a”

category which 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 treatment showed a faster movement compared to control. The treatment using 25% of lemon extract provided the fastest motility compared to other condition showing that 25% of lemon extract providing high energy for sperm to do the activities.

The other possible reason of high motility of sperm by treating with 25% of lemon extract was the sperm morphology. The progressive quality of sperm motility occured if the sperm have a normal shape. The morphology of normal sperm was showed by the perfect tapered head and hooked ends, followed by a straight neck and a long, non-bent tail. The sperm like these are observed for concentration and morphology and which 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 high quantity of normal shape of sperm morphology. The number of normal sperm morphology decreased when the lemon extract concentration increase, showing the high concentration of lemon extract provided the negative impact in the spermatozoa and affected the sperm morphology (Ferial & Muchlis, 2013; Nirmalasari, 2017).

The role of vitamin C in the spermatozoa have been tested by treating the patient who underwent the varicocele surgery. The patient was given with 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 the sperm quality and quantity was begun during the period of maturation in the epididymis. The vitamin C support the sperm development as an additional energy which ensure the successfully of spermatozoa process. The other role was an actioxidant where the vitamin C containing in the lemon extract protected the sperm from free radicals which potentially disturb the spermatozoa process (Noorul et al., 2017). The free radicals, also known as Reactive Oxygen Species (ROS), were the substances which could inhibit the sperm development because of their free ion radical. The presence of vitamin C as the antioxidant stabilized the free ion radical to perform the neutral substances which effectively used in the sperm development. However, the high amount of ROS sometimes was higher than the amount of antioxidant and initiated the cells experienced the oxidative stress leading to cell damage (Nicol & Prasad, 2006). On the other hand, the high amount of antioxidant also performed the negative impact where the exceeds of antioxidant could not be an 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 the treatment of lemon concentration 50% was not followed by an increase 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 as the primary abnormalities, or at the stage of improvement in the epididymis called as 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, there was no comprehensive data showing 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. The minerals together with Na and Zn were essential minerals because they are components of various important enzymes. Therefore, suboptimal concentrations of these minerals are associated with male infertility. For example, Calcium could trigger for acrosome reactions which supported the sperm motility (Harchegani et al., 2019; Halo et al., 2018). On the other hand, the concentration of potassium (K) was related to the production of testosterone, while the amount of calcium content affected the increase of slow-moving and immotile sperm (Hamad et al., 2014).

Conclusion

The roles of lemon extract to support the sperm development was complex where each element such as vitamin C, minerals, and the other vitamin containing 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 exceeds amount of lemon extract could not provide an additional function but showing the opposite effect which could debilitate the function of lemon extract treatment.

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