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THE EFFECT OF CO-REARING WITH FEMALES ON THE QUANTITY AND QUALITY OF MOUSE (*Mus musculus*) SPERMATOZOA

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ABSTRACT
Coexistence with the same sex is often discussed in recent times, but its effect on reproductive health has not been widely publicized. This study used male (*Mus musculus*) animals to determine the effect of co- rearing female animals on the quantity and quality of spermatozoa. The research type carried out was purely experimental with a completely randomized design with 4 treatments, namely control (P0) rearing which contained only 5 male mice, P1 rearing consisting of 3 males and 2 females, and P1 rearing consisting of 3 males and 2 females, P2 consisting of 2 males and 3 females while P3 contained 1 male and 4 female. Each treatment consisted of 6 replications. Animals are kept from 4 weeks old to 10 weeks old. Parameters observed are concentration, motility, and normal morphology of spermatozoa, and data analysis was tested by Kruskall-Wallis Test followed by Duncan's test. the best spermatozoa compared to other treatments. The results showed that there were significant differences in the quantity and quality of spermatozoa, in the various treatment and maintenance groups, which indicated that the treatment with the composition of 1 male and 4 females had the best quantity and quality of spermatozoa compared to other treatments.

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1. INTRODUCTION

In social life, interaction between individual and group will have a certain influence. Over time, this influence includes physical changes marked by the emergence of the reproductive organ development and maturation process (Munawaroh, 2001). This condition will encourage each individual to establish variation in sexual orientation (Kusmiran, 2011) for example, attraction to the opposite sex or even being attracted to individual of the same sex. (Azhari and Kencana, 2008). Social interaction also affects mental health, namely stress (Ono et al., 2011). This is in accordance with Goliszek's (2005) statement that stress is a consequence or result of environmental situation cause psychological and physical demands on a person due to social interactions. Furthermore, stress is also one of the factors that can cause infertility Tai (2013). In this case, problems with the male reproductive system can have an impact on difficulty in breeding (Fadilah et al., 2017).

The result of the study prove that psychological stress is related to the quantity and quality of spermatozoa (Matthew et al., 2002). If stress occurs continuously, it will cause a decrease in reproductive function which can cause infertility (Fenster et al., 1997). This is because stress will stimulate the hypothalamus to produce Corticotropic Releasing Hormone (CRH) which causes the release of Adreno Corticotropin Hormone (ACTH) in the pituitary. The release of ACTH will stimulate the adrenal cortex to release cortisol (Wade, 2007). A continuous increase in CRH will cause a decrease in GnRH and the production of follicle stimulating hormone (FSH), luteinizing hormone (LH) by the pituitary so that it will interfere with the process of spermatogenesis and affect the quality of spermatozoa (Selvage and Rivier, 2003).

Physical and psychological stress is a stress that can activate the endocrine system central and peripheral response. The response to stress involves the activity of the sympathetic nervous system and the Hypothalamic

Pituitary Androgen (HPA) axis (Qi et al., 2016). The Hypothalamic Pituitary Gonadal (HPG) axis has a number of component structures and neural circuits to the HPA axis, and these two axes work to maintain a regulatory balance. The relationship between them is reciprocal. Several studies have found that under stress condition there will be suppression of the reproductive system, increased glucocorticoids will suppress GnRH and LH and result in a decrease in gonadal hormone secretion (Saxbe, 2015).

The result of the observation on male mouse (Mus musculus) animal kept with female mouse showed that male individual carried out allogrooming activity to female mouse or to other male Oktiansyah (2015). Allogrooming is a physical activity (touch) carried out by at least two individuals of the same or different sex in one group with the aim of strengthening individual relationship between groups and easing tension between individuals during conflict (Matheson & Berstein, 2000).

Meanwhile, research on the growth of Oreochromis aureus fish reared with different male and female ratios showed that the composition of 100% males experienced the best growth compared to other compositions (75% males 25 females, 50% males 50 females) even the composition 25% males 75% females experienced the slowest growth . (Robisalmi et al, 2017). Based on the last two studies above, there are no data showing the reproductive effect of co- rearing male and female individuals. Thus, this study aims to determine whether co-rearing with females affects the quantity and quality of male mouse (Mus musculus) spermatozoa.

2. RESEARCH METHOD

This research is a pure experimental study (True Experiment) using a completely randomized design with 4 treatments and 6 replications each. The control treatment (P0) is the maintenance of 5 male mice only. This number refers to the Mus musculus maintenance provision in one cage with a maximum of 5 mice. The next treatment is P1, keeping mice in one cage consisting of 3 male mice and 2 female mice and P2 consisting of 2 male mice and 3 female mice. While P3 contains 1 male mouse and 4 female mice. The total number of mice used were 66 male mice and 54 female mice.

Maintenance Step. Mice aged 4 weeks (average body weight of 10-11 g) were acclimatized for 1 week, then placed in cages with predetermined treatment. Plastic tub cage with a size of 30 x 20 x 17 cm, with sawdust base and wire cover. Mice were reared until they were 10 weeks. During the treatment, the mice were fed pellets and drank aqua water ad libitum.

Spermatozoa Observation Step. After the mice were 10 weeks old, all male mice were killed by neck dislocation to collect spermatozoa. The scrotum was dissected and the epididymis was cut into pieces, put into a petri dish containing 0.9% physiological NaCl, stirred with a glass stirrer until homogeneous. One drop of homogeneous suspension (10 µL) was placed on top of the Neubauer counting chamber for concentration and motility observations, while the morphology of spermatozoa was observed using a slide. Observations on concentration, motility and morphology were based on WHO (2010) standard and used a multimedia microscope.

The concentration of spermatozoa is calculated with the condition that if there are less than 10 spermatozoa in one box, then 25 boxes are counted, if it contains 10-40 then 10 boxes are counted, while for those containing more than 40, only 5 boxes are counted. The microscope magnification used was 40 x 10. Spermatozoa concentration (million/ml) = Number of sperm in all chambers x dilution factor x 0.05 x 106 (Karimah, 2017). Almost the same as the concentration the spermatozoa motility observed is spermatozoa movement which included fast and forward movement (progressive = category a), slow moving or circular (non-progressive = category b) and immobile (immotility = category c). Spermatozoa motility is expressed as a percentage by dividing the movement of spermatozoa category a+b with category a+b+c multiplied by 100%. (Nisa'ina, 2015)

Specifically for the morphology of spermatozoa, it must first make a smear before making observations by placing one drop of suspension on a slide, buffing it off and allowing it to dry. After drying, they are soaked in absolute methanol for 5 minutes, then rinsed with distilled water and dried. The next preparation, it is soaked again in safranin for 5 minutes, and immersed in the Phosphate Buffer solution three times. The last step, the preparation is dipped in a crystal violet solution for 5 minutes, washed with running water and allowed to dry (Hanizar, 2004). The preparations are observed under a microscope with a magnification of 1000 times. Normality of spermatozoa is assessed from the condition of the head, neck and tail after observation of 100 spermatozoa.

Spermatozoa of mouse is normally shaped if the head has a tapered hook, the neck is clear and the tail is long and straight. Normality of spermatozoa is calculated from the number that have normal quality divided by all observed spermatozoa multiplied by 100%.

Data analysis. All data are analyzed using the Statistical Package for Social Science (SPSS) version 22. The normality test use the Kolmogorov-Smirnov Test, while the homogeneity test use the Levene Test. The data are further analyzed by using the non-parametric Kruskall-Wallis test to determine whether there are differences in the treatment with females on the concentration, motility and morphology of mice spermatozoa. Duncan's further test is conducted to determine the differences between treatments.

3. RESULT AND DISCUSSION

Results

The result of observation of female co-maintenance on the concentration of mouse spermatozoa (Mus musculus) are presented in table 1. The normality test showed that the data is not normally distributed ($p < 0.05$), as well as the homogeneity showed that the data is not homogeneous ($p < 0.05$). Thus, the data is analyzed using the Kruskall-Wallis test. The result of the analysis showed that there are differences in female co-rearing between treatment and control on the mice spermatozoa concentration ($p < 0.05$). Furthermore, Duncan's test to compare the average of each treatment is shown in table 1

Table 1. The average concentration of spermatozoa (millions/mL) in each treatment from Duncan's test result

Group	Average (million/ml) ± SD
P_0 = 5 Male Mice *(Mus musculus)*	38,067 ± 11,942[a]
P_1 = 3 Male Mice + 2 females Mice *(Mus Musculus)*	68,661 ± 25,385[b]
P_2 = 2 Male Mice + 3 females Mice *(Mus Musculus)*	94,933 ± 14.537[d]
P_3 = 1 Male Mouse + 4 females Mice *(Mus Musculus)*	80,322 ± 12,964[c]

Averages followed by different notations show significantly different results

In table 1 it can be seen that the mean concentration of spermatozoa at P1, P2, and P3 is significantly different from the control treatment (P0). Likewise, P1 is different from P2 and P3, and P2 is different from P3. The result of the normality test of spermatozoa motility data showed that the data are not normally distributed ($p < 0.05$) and not homogeneous ($p < 0.05$) so that the data are further analyzed using the Kruskall-Wallis test. The result of the analysis showed that there are differences in female co-rearing between the treatment and control of the motility of the mice's spermatozoa ($p < 0.05$), Duncan's test result showed the differences between the treatments are shown in table 2.

Table 2. The average sperm motility of mice (%) Duncan test results

Group	Average (million/ml) ± SD
P_0 = 5 Male Mice *(Mus musculus)*	66,2 ± 45,837[a]
P_1 = 3 Male Mice + 2 females Mice *(Mus Musculus)*	87,3 ± 87,088[b]
P_2 = 2 Male Mice + 3 females Mice *(Mus Musculus)*	86,8 ± 89,815[d]
P_3 = 1 Male Mouse + 4 females Mice *(Mus Musculus)*	93,2 ± 61,654[c]

Table 3. Average morphology of normal spermatozoa (%) Duncan test result

Group	Average (million/ml) ± SD
P_0 = 5 Male Mice *(Mus musculus)*	85,67 ± 4,802[a]
P_1 = 3 Male Mice + 2 females Mice *(Mus Musculus)*	88,33 ± 3,710[b]
P_2 = 2 Male Mice + 3 females Mice *(Mus Musculus)*	88,44 ± 3,650[d]
P_3 = 1 Male Mouse + 4 females Mice *(Mus Musculus)*	90,83 ± 2,503[c]

In contrast to the condition in response to the concentration of spermatozoa, the treatment of P1 (3 males + 2 females Mus musculus) and P2 (2 males + 3 females Mus musculus) do not show any difference. While the P3 treatment (1 male + 4 female Mus musculus) is different from the control.

Similar to the concentration and motility data, the result of the normality and homogeneity test of spermatozoa morphology data also showed that they are not normally distributed ($p < 0.05$) and they are not homogeneous ($p < 0.05$). Therefore, the data are analyzed using the Kruskall-Wallis test, and the result showed that there are differences in female co-maintenance on the morphology of mouse spermatozoa ($p < 0.05$). Duncan's further test to compare the mean of each treatment is shown in table 3.

The table above shows that the treatments of P1 (3 Males + 2 Females Mus musculus), P2 (2 Males + 3 Females Mus musculus), and P3 (1 Male + 4 Females Mus musculus) are not significantly different, although significantly different from the control treatment.

Discuccions

The quality and quantity of spermatozoa as a response to male and female rearing are observed microscopically with 3 parameters, namely concentration, motility, and normal morphology of spermatozoa. The results of statistical analysis showed that the co-rearing treatment of females showed an increase in the concentration, motility and normal morphology of mouse spermatozoa.

The concentration of spermatozoa produced depends on the process of spermatogenesis in the seminiferous tubules. Based on research (Ramadhani, 2007) that the normal number of spermatozoa in mice is ± 50 million/mL. If there is a disturbance in the process of spermatogenesis, the development of spermatozoa cells will be disrupted so that it can affect the concentration of spermatozoa formed. On the other hand, if spermatogenesis proceeds normally, the concentration of spermatozoa produced will be normal, and there may even be an increase in spermatozoa.

The result of this study showed the concentration of spermatozoa in the control treatment, namely 5 males had a low spermatozoa concentration (38.067 ± 11.942) and it below the average number of normal spermatozoa in mice. This is because the group experienced stress in response to same-sex rearing. The behavior that appears from the activity of the male mice is licking and riding on another male's body.

In accordance with Oktiansyah's research (2015), that male mice ride other mice as a genital examination to test suitability and detect reproductive organs of the opposite sex, both male and female. This condition is supported by Fitzpatrick (2014) which states that same-sex marriage interaction will complicate effort to determine sperm quality and affect male fertility. Meanwhile, the result of research by Matthew et al, (2002) showed that psychological stress in mice can cause hormonal disturbance, resulting in the failure of Leydig cells to secrete the hormone testosterone.

As explained above, stress condition will stimulate the hypothalamus to secrete CRH which in turn causes ACTH secretion in the pituitary. The release of CRH, on the other hand, suppresses the release of GnRH and LH, thereby suppressing the reproductive system. Thus, the low mean concentration of spermatozoa in treatment of 5 males occurred due to decreased secretion of hormones involved in spermatogenesis, namely LH, FSH and testosterone. Lack of this hormone will inhibit the process of spermatogonia proliferation and will eventually interfere with the process of spermatogenesis.

(Erris and Harahap, 2014).

In the treatment 2 Males Mice + 3 Females Mice had the highest average concentration above normal spermatozoa, namely 94,633 million/ml ± 14.537. This condition is supported by the results of research by Gasparin et al., (2009) that the presence of females can change the quality of spermatozoa due to libido activity. Rachmawati et al., (2014) also concluded that the higher the libido level, the higher the testosterone level and the better the sperm quality.

Meanwhile, in the treatment 3 males' mice + 2 females' mice had a low mean number of spermatozoa (68.661 million/ml ± 25.385) compared to the treatment 2 males' mice + 3 females' mice (94.633 million/ml ± 14.537), and 1 male mouse + 4 females' mice (80.322 ± 12,964). This is because the ratio of males is more than females which causes competition between males and dominance occurs, causing psychological stress in male mice. The behavior describes the competitive activity of these mice when a pair of male and female copulation is prevented by other males' mice and females' mice. Based on observations carried out by Indah et al., (2014), male animal will separate and even protect their female from other male and female animal, so that the dominant male prevents the copulation of the less dominant male. In accordance with the research of Birkhead et al., (2009) stated that female mating with multiple males cause the resulting spermatozoa to be incompetent enough to fertilize.

While in the treatment 1 Male + 4 Females had a lower spermatozoa concentration (80.322 ± 12.964c) than 2 males + 3 females (94.633 million/ml ± 14.537) because there was no competition between males in the group, so males freely exercised frequency relationship with any female. Individual who frequently has sex will have reduced fertility because the spermatozoa have not yet had time to mature so they cannot fertilize the egg (Rudiyat, 2018).

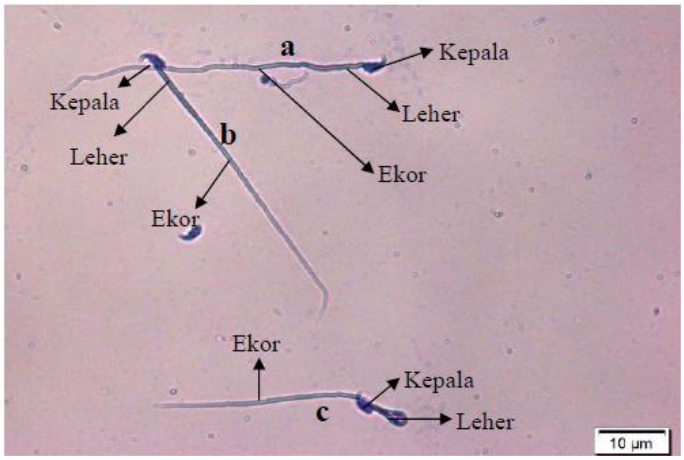
Spermatozoa motility is one of the important indicators in determining the quality of spermatozoa (Elzanaty and Malm, 2007). The motility in question is the progressive movement of spermatozoa, namely spermatozoa move forward and fast or move slowly because this category of movement allows spermatozoa to reach the ovum. Spermatozoa motility is supported by Adenosine Triphosphate (ATP) which is formed by oxidative phosphorylation in mitochondria located at the base of the tail of spermatozoa. High ATP content can provide more energy so that it can increase the movement of spermatozoa (Cummins, 2009). Based on statistical analysis, it was found that co-rearing of females gave different spermatozoa motility responses compared to controls. In this treatment (5 males) had low spermatozoa motility (66.2 ± 45.837) because maintenance of the same sex resulted in various activities that trigger physical and psychological stress, including fighting, making out, and riding other males to test suitability. Marry. This stress causes a decrease in male reproductive function, one of which is a decrease in sperm motility (Clarke, 1998).

Meanwhile, the motility of spermatozoa in the treatment of 3 males + 2 females (87.3 ± 87.088) and 2 males + 3 females (86.8 ± 89.815) showed no difference. This is because in the treatment there is competition in terms of marriage. Seen in the activity of male mice when they want to have sexual relations with females, jealousy and interference by other males and females occur so that copulation does not go well, causing stress. In contrast to the treatment of 1 Male + 4 Females (93.2 ± 61.654), where the male animals did not experience physical stress due to competition or there were no barriers to sexual activity at any time.

The result of this observation is in accordance with Ratomo's research (2014) that stress can trigger the release of steroid hormones or glucocorticoids which can blunt testosterone levels thereby reducing spermatozoa motility. In addition, Simmon and Fitzpatrick (2012) also stated that sperm motility is better when under non-competitive conditions or there is no competition.

In the same direction as the concentration and motility parameter, the morphological observation of normal spermatozoa also showed a different response from the control. The result of the observation showed that 5 males (85.67 ± 4,802) had a low mean of normal morphology because maintenance of the same sex caused oxidative stress which resulted in a decrease in cell membrane integrity resulting in abnormal morphology in spermatozoa.

In contrast to the treatment of 3 males + 2 females (88.33 ± 3.710), 2 males + 3 females (88.44 ± 3.650), and 1 male + 4 females (90.83 ± 2.503b) there is no significant difference. These three treatments have normal spermatozoa counts above the control treatments. However, there are spermatozoa looked abnormal, namely the position of the body, precisely in the folded neck area (Figure 1). While other normal spermatozoa show a head equipped with a tapered hook and a position from the neck to the tail is straight.



Picture 1. Spermatozoa morphology of male mice (Mus musculus), after staining with Safranin and Crystal Violet. Description: a, b = Normal Spermatozoa Mice (Mus musculus), c = Abnormal Spermatozoa Mice (Mus musculus). (450X Magnification)

The number of normal spermatozoa in the treatment is due to the low stress so that the levels of hormones involved in spermatogenesis do not interfere with the spermatozoa maturation process. The percentage of normal spermatozoa morphology is more common in all treatments than abnormal spermatozoa. If you look at the normal morphology of spermatozoa from this study, which is > 85%, it is still included in the category according to WHO standard.

4. CONCLUSION

The presence of female individual in the maintenance of male mice affects the concentration and quality of the spermatozoa produced. The greater the ratio between male and female individual, the higher the concentration and motility of spermatozoa, except for the normal morphology of spermatozoa.

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