

The effectiveness of ^{60}Co gamma-ray exposure to the reproductive systems of rat (*Rattus argentiventer*) as sterile male technique

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Abstract. Sutapa GN, Supartha IW, Wijaya IN, Puja IK, Syaifudin M. 2020. The effectiveness of ^{60}Co gamma ray exposure to the reproductive systems of rat (*Rattus argentiventer*) as sterile male technique. *Biodiversitas* 21: 3805-3810. Various strategies have been implemented to control pests, however, these do not able to reduce rat attacks on rice plants. Therefore, the sterile male technique (SMT) using ^{60}Co gamma radiation was introduced. A group of rats (*Rattus argentiventer*) of 2 months age were irradiated with 1-6 Gy doses (dose rate of 99.5631 cGy/min) and the quantity, morphology, and viability of spermatozoa of rats were assessed with standard procedures. The results showed that the concentration of spermatozoa was decreased with increasing radiation doses where for lower than 4 Gy were categorized as normal (>20 million/mL). The morphology of spermatozoa also decreased with increasing radiation dose where under 3 Gy were in normal (> 50%). Spermatozoa viability was also decreased where for doses 1 and 2 Gy were categorized as normal. Statistical analysis showed a significant difference ($p < 0.05$) between the exposed group to control in spermatozoa quantity, morphology, and viability. This finding is quite similar with other experiments on the reproductive appearance of rat based on spermatozoa after irradiation. It is concluded that dose of 3 Gy that caused changes in spermatozoa with its viability still above 50% is the most appropriate dose for SMT of rice field rats.

Keywords: ^{60}Co gamma radiation, rat pest, reproductive system, sterile male technique

INTRODUCTION

Rice field rats (*Rattus argentiventer*) are one of the main pests of rice plants, which have different properties from other main types of pests. They can attack all stages of rice plants, either at the vegetative, generative, or post-harvest stages (Sudarmaji and Herawati 2017) and significant damage to agricultural production throughout the world. In Indonesia, the level of damaged rice field caused by rodents attack reached 161000 ha/year in 2015. This is equivalent to the loss of 555 million kg of rice, which is enough to feed around 6.3 million residents for one year (Sudarmaji and Herawati 2017). In 2010-2017 rice field pest attacks have caused the loss of 12,013 ha of rice in Bali Province with an increased rate of damage reaching 2403 ha/year. The area with the most damage was Tabanan Regency, reaching 6,061 ha or 1,212 ha/year (BPTPH 2017).

Rat attack can cause damage to generative stadia of rice plants that result in fatal damage because rice can not compensate for damage with the formation of new tillers. This damage may cause significant economic losses (Singleton et al. 2010) and the rat pests have an important meaning in rice farming (Brown et al. 2017). Department of Agriculture of Bali Province in 2017 reported that the number of rats that were captured for 2 days hunting has

reached 158048, of which 67.7% was captured in Tabanan Regency. The attack of rice field rats ranks first as a plant disturbing organisms (PDO) compared to other pests such as rice stem hoists, tungro disease, brown planthopper (*Nilaparvata lugens*), and blasts (Wisnuwardana 2010).

Various technologies have been carried out to control rat pests such as narrow bunds, rat traps, doing crops and harvesting simultaneously, improvement of sanitation, crop rotation, rodenticide or poison bait, and rat predators (Belmain et al. 2015; Sekarweni et al. 2019). Ecological control (Baco 2011), predators (Prakash 2018), trap barrier system, and linear trap barrier system (Kabir and Hossain 2014; Brown et al. 2017) have also been done. The use of rodent control technology did not effectively prevent a vitriolic attack on rice crops (Wisnuwardana 2010; BPTPH 2017). Therefore it is necessary to introduce the sterile male technique (SMT) as one of the components of the integrated pest control system.

The SMT was introduced by Knippling (1955) to control livestock flies (*Cochliomyia hominivorax*). Bellini et al. (2013) used SMT with irradiation to control *Ae. albopictus* mosquitoes and revealed a significant sterility level in the local population. Maiga et al. (2014) reported that gamma irradiation at dose of 90 Gy to *An. coluzzii* pupae result in the index of mosquito breeding competitiveness of 0.53. In rodent control, more than half-century ago La Chanca et al.

(1967) has carried out the SMT in rats using X-rays. Nahar et al. (2006) have also carried out a similar experiment by fast neutrons, where the effect of infertility or sterilization had occurred at a dose ranging from 40 to 200 rad for 25 weeks. Research on the effect of X-ray radiation on spermatozoa of rat conducted by Hariyoto et al. (2002) showed that X-ray radiation exposure decreases sperm motility and viability in Wistar rats with the optimum doses 100 mGy. According to Biedka et al. (2016), a radiation dose of 0.15 Gy caused a temporary threshold dose of sterility, whereas a threshold dose for permanent sterility in male rats is 3 Gy (Jones et al. 2019). However, there is still limited data on the dose of gamma rays for sterility of rice field rats. This study aims to determine the effect of Cobalt-60 gamma-ray irradiation on the fertility of male rice field rats by mentioning the quantity, morphology, and viability of spermatozoa.

MATERIALS AND METHODS

Research design

This type of research was experimental, that is, the first experiment was designed to determine the effect of Co-60 gamma radiation doses on the reproductive system appearance of male rice field rats, which includes concentration, morphological, and viability of the spermatozoa. The experiments used a randomized block design, which consisted of 7 groups with 6 treatment groups of Co-60 gamma radiation and a control group.

Ethics

The protocol used in the experiments had been approved by the Governmental Ethics Committee Review Board of Research and Development Unit, Faculty of Medicine, Udayana University with the number of 695/UN.14.2/Litbang/2013.

Radiation process

Before the research was carried out, rats were grouped based on the sex of adult rats that were ready to be mated (female at aged 40 days and male at age 60 days). Irradiation was done with a surface to source distance (SSD) was 80 cm on a 20 x 20 cm² field, and conducted at the Sanglah Hospital Denpasar Radiotherapy Installation. The whole-body radiation of male rats was carried out with a Co-60 FCC 8000F Teletherapy device at a dose rate of 99.5631 cGy/min. The variation doses were 0, 1, 2, 3, 4, 5 and 6 Gy that each radiation time was done for 0, 57, 115, 172, 229, 287 and 344 seconds. The radiation dose was calculated at a maximum depth of 0.5 cm (according to the reference of energy of ⁶⁰Co) (Karbalaei et al. 2017), whereas the maximum depth and width of the field was 20 x 20 cm².

Reproductive system preparation

After irradiation, rats were sacrificed by dislocation of the neck, then surgery was performed on the lower abdomen and this was done at the Reproduction Laboratory of the Animal Hospital of Udayana University in Denpasar,

Indonesia. The testes and epididymis were taken and put in a physiological saline solution (0.9% NaCl) and then cauda epididymis was separated from the testes and the attached fat was cleaned, then an epididymis of 0.5 cm of size was chopped in 1 mL of a physiological saline solution using scissors and was dissected until a spermatozoa suspension was obtained.

Spermatozoa concentration

The spermatozoa concentration was calculated using an Improved Neubauer booth (hemocytometer). Ten microliters of spermatozoa suspension were taken and then placed into the counting chamber. A hemocytometer containing a spermatozoa suspension was then observed under a light microscope with a magnification of 400x with 10 replications of observation for each sample. The hemocytometer chamber consists of 4 chambers: top left and right, bottom left and right. Each room consists of 16 small boxes (square). The calculated spermatozoa concentration is the average concentration of spermatozoa in these 4 chambers denoted by the letter L. The calculation of spermatozoa concentration per mL was as follows (Doucette 2020): the volume of each square = $1/4 \times 1/4 \times 1/10 = 1/160 \text{ mm}^3$, the volume of each room = $16 \times 1/160 = 0.1 \text{ mm}^3 = 0.1 \mu\text{L} = 10^{-4} \text{ mL}$, so for every 1 mL it should be times with 10^4 . Quantity of spermatozoa (cells/mL) = $L \times 10^4 \times \text{dilution}$, which is stated in million/mL, was divided into the following criteria: as normal if the number of spermatozoa is ≥ 20 million/mL, as suspect if the number of spermatozoa is 10-20 million/mL, and as abnormal if spermatozoa count is <10 million/mL.

Spermatozoa morphology

Morphological observations were carried out by making a sperm smear. One drop of spermatozoa was put on a clean glass slide and a drop of 1% eosin and a drop of 10% nigrosine were added. After the smear was air-dried then it was viewed under a microscope, the number of the normal forms of random collection per 100 spermatozoa were counted. The morphological examination includes the completeness of the head and tail of spermatozoa with the following criteria: normal if the morphology is $\geq 50\%$ normal, suspect if the morphology is 40-50% normal, and abnormal if the normal morphology is $<40\%$ (Khatun et al. 2018).

Spermatozoa viability

To observe the viability, smeared spermatozoa were stained with 1% eosin and 10% nigrosin dyes. The viability of spermatozoa is observed under a light microscope at a magnification of 400x. Red spermatozoa indicated as dead whereas colorless (transparent) spermatozoa indicated as still alive. Calculation of spermatozoa viability was performed in 100 spermatozoa cells and then expressed as a percentage of spermatozoa that were still alive. The viability of spermatozoa was properly functioning if it has an amount above 40% (Khatun et al. 2018).

Data analysis

The statistical analysis used was the analysis of variance, using the SPSS (Statistical Product and Service Solutions) program for Windows version 23. To check the significant difference of Co-60 gamma radiation to the concentration, morphology, and viability of spermatozoa was at the level of 0.05. If there is a significant then it continued with the Mann-Whitney Test.

RESULTS AND DISCUSSION

In this study there was an increase in the number of pups born from a generation to the next generation of rat maintained in the animal laboratory, with an average of 2 pups in the first generation (F1) to 9 pups in the third generation (F3) (Figure 1). This 3rd generation of rat (F3) was used for the experiment, of which they had pattern and normal breeding abilities as the condition in the fields similar to their natural habitat.

The results showed that the concentration of spermatozoa was decreased with increasing radiation doses. These were 145, 100, 62 and 25 million/mL for 1, 2, 3 and 4 Gy exposed rat, respectively, and all were categorized as normal concentrations (>20 million/mL), whereas normal concentration of spermatozoa (the control group) was 158 million/mL. For the doses of 5 and 6 Gy, the amounts of spermatozoa were below 10 million/mL and were categorized as an abnormal concentration. The concentration, morphology, and viability of spermatozoa of mouse after irradiation are presented in Table 1.

The results showed that the morphology of spermatozoa was decreased with an increase of radiation dose, these were to be 92.22; 80.30; 57.20% of control (0 Gy) for 1, 2, 3 Gy, respectively, and still included as normal morphology (>50%). Whereas for the 4, 5, and 6 Gy doses these were to be very low (20.90%; 0% and 0%, respectively) which means these were under 40% and categorized as abnormal morphology. Some observations of reproductive system appearance under an optical microscope at magnification of 450 times are shown in Figure 2.

Viability of spermatozoa was decreased to be 72.26; 58.96 and 51.98% of control for doses of 1, 2, and 3 Gy and still normal, whereas for 4; 5; 6 Gy their viability were

17.8; 0; 0%, respectively and included as abnormal. Statistical analysis results rat showed a significant decrease in spermatozoa concentration and their morphology and viability due to all radiation dose treatments compared to control with a probability value of $p < 0.05$.

Rodents remain one of the main nuisances to mankind and considered major pests because of their devastating impacts. They have been causing damage to crops, stored grain, and infrastructure. They grow very fast due to their natural behavior as many offspring in once birth and short gestation period. However, it is quite difficult in maintaining them in laboratory. In this study, the 3rd generation of rats was used as experimental animal. This is the same as the average number of pups per litter at birth by rat in their habitat or in experimental cage, which is around 10-13 pups per generation (Pritchett-Corning et al. 2009; Allen et al. 2013).

The reduction of spermatozoa number due to radiation found in this study is similar to the results of experiment by Gong (2014) where the 2 Gy radiation dose significantly reduces the number of spermatozoa. The decrease in spermatozoa cell number occurred due to damage to the testes and epididymis which is very dependent on the total dose of radiation delivered. For doses of 5 and 6 Gy, Biedka et al. (2016) stated that there was no decrease in the quality of spermatozoa because this dose had caused highly damaged spermatozoa cells, as found in this research.

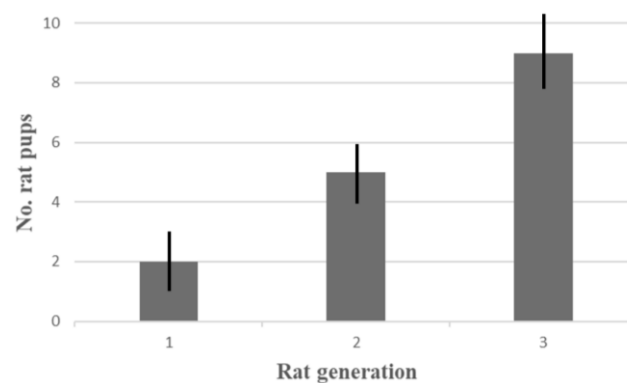


Figure 1. Number of rat pups born in each generation where the 3rd generation was used in the experiment

Table 1. The number and percentage changes in morphology and viability of rice field rat reproductive systems in various radiation dose variations

Reproductive system (spermatozoa)	Control (0 Gy)	Radiation dose (Gy)					
		1	2	3	4	5	6
Concentration (million/mL)	158.00 ± 1.41	145.00 ± 1.58	100.00 ± 1.58	62.00 ± 1.58	25.00 ± 1.00	0	0
Morphology (%)	99.89 ± 0.03	92.22 ± 0.45	80.30 ± 0.49	57.20 ± 2.20	20.90 ± 0.20	0	0
Viability (%)	96.96 ± 0.24	72.26 ± 1.78	58.96 ± 0.63	51.98 ± 1.28	17.80 ± 0.82	0	0

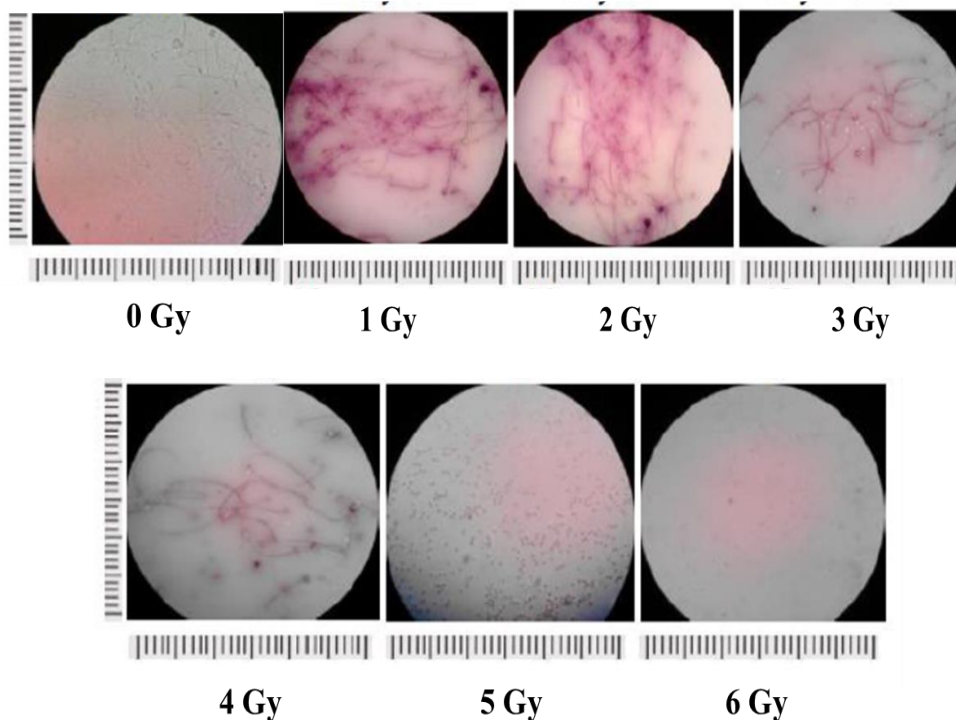


Figure 2. Microscopic visualization of rice field rat reproduction appearance at various dose of whole-body gamma radiation

Radiation greatly affects sperm morphology, namely the formation of DNA damage in the reproductive system including chromosomal aberration, gene mutations, and the process of germinal cell apoptosis (Khan et al. 2015). Gamma radiation easily interacts with spermatozoa which composed of 95-98% water originating from the prostate gland and seminal vesicles. Changes in the concentration of hydrogen ions due to the interaction of gamma-ray photons that ionize water molecules in spermatozoa, thereby increasing the production of H^+ and OH^- ions. Changes in H^+ and OH^- ion concentration will result in changes in sperm pH and can cause morphological changes (Zhou et al. 2015). For direct effects, it can be considered that radiation exposure would result in persistence of DNA damage that leads to down-regulation of DNA repair and loss of apoptotic capabilities during spermatogenesis (Fuller et al. 2019). Meanwhile, according to Bonato et al. (2012) and Zhou et al. (2015) the normal pH of sperm is 7-8 so that more acidic or more alkaline will affect viability of sperm cells. In addition to the influence of pH, environmental temperatures that are too low or too high can affect the reproductive organs of male rats. This disrupts the function of scrotal thermoregulatory failing spermatozoa formation and decreased spermatozoa production. Reproductive organ males placed in hot rooms have a low fertility rate that caused by deterioration in the quality of semen and 10% of abnormal spermatozoa (Chirault et al. 2015).

Payne (1994) demonstrates that exposure to rats at dose of 1-4 Gy would result in increase in the frequency of apoptosis in germline cells causing morphological alteration and azoospermia. This change causes a decrease

in normal sperm morphology with an increase in the rate of defective sperm in the head or tail. Research conducted by Adamkovicova et al. (2016) found that an increase in radiation dose given to rats would cause an increase in spermatozoa with abnormal morphology of head and tail. In accordance with the results of the research proposed by Makinta (2005) and Adamkovicova et al. (2016) that radiation doses cause excess estrogen, high levels of estrogen that negatively affect the internal environment of the epididymis, resulting in stiff sperm and flagellum bending the tail of spermatozoa. Our results also demonstrated that radiation caused the increase in number of sperms with the defect in head and tail. Further, it would cause infertility. For doses of 5 and 6 Gy there was no decrease in spermatozoa morphology because this radiation dose had damaged spermatozoa cells. It was predicted that this high dose radiation caused a considerable reduction in sperm quantity and quality, where plasma membrane integrity is known to be sensitive to free radicals (Fuller et al. 2019). Likewise, the Mann-Whitney test showed a significant change between the control and the 1-4 Gy treatment. A decrease in normal sperm morphology due to an increase in the rate of sperm with head or tail defects was found after exposure to radiation doses above 1 Gy (Adamkovicova et al. 2016).

The underlying molecular mechanisms of low-dose radiation-induced risks for spermatogenesis remain unclear. However, it is predicted that radiation greatly affects the concentration of sperm through free radical interactions that are formed as well as direct interactions that cause DNA damage (Ford et al. 2012; Fuller et al. 2019). The deterministic effect on the reproductive organs or gonads

can interfere with the formation of spermatozoa produced. Kesari et al. (2018) stated that radiation harms reproductive aspects such as spermatogenic cell populations and cell malformations, which causes a reduction in the number of sperm and non-living spermatozoa. Radiation doses as low as 0.15 Gy could result in decreased concentrations of spermatozoa (oligospermia) (Jones et al. 2019). Decreased spermatozoa concentration could be affecting fertility. One factor that can affect fertility is radiation exposure, especially ionizing radiation (X-rays and gamma rays) (Olayemi 2010). Moreover, male fertility can be readily impacted by exposure to radiation, even to the chronic exposure such as environmental dose (Fukunaga et al. 2017).

Observation of viability or percentage of living spermatozoa is done by staining using eosin solution. Red color means death sperm, and colorless means life (Agarwal et al. 2016). Eosin dye cannot enter the living spermatozoa cells, because the plasma membrane of living spermatozoa is intact or has not been damaged (Farah et al. 2013).

The results of this study showed that the viability of spermatozoa in control (0 Gy) rat reached 96.96%, whereas, at doses of 3, 4, 5 and 6 Gy spermatozoa were functionally unlikely to be able to move because the mitochondria contained in the tail midpiece were no longer producing energy. Even though sperm could not move in rat exposed to 3 Gy radiation, its viability still reached above 50% and it still can fertilize. Sperm that are not motile but still have viability (viability above 50%) can be used as artificial insemination (AI), according to the results of research conducted by Nagata et al. (2019). The results are similar to the results of a study by Marci et al. (2018) where a decrease in the gonad system occurs with increasing radiation doses. This decrease includes sperm quality which includes sperm count, normality or morphology, motility, and viability or endurance. This study also shows the effect of giving a dose of gamma-ray radiation on sperm concentration which also determines the quality of sperm. A dose of as low as 100 mGy (0.1 Gy) has caused a decrease in concentration of sperm.

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