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Effect of Green Tea Extract on Spermatozoa Quality of Peranakan Ongole Bull On Frozen Storage

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Abstract. Low-temperature storage can often result in spermatozoa damage. It occurs due to Reactive Oxygen Species, leading to changes in the lipid composition of the membrane. Antioxidant compounds are needed to prevent ROS. Green tea extract (*Camelia sinensis*) can be used as an antioxidant agent. This study aimed to determine the effect of green tea extract in tris egg yolk on spermatozoa quality of Ongole Peranakan (PO) cattle. This study used a completely randomized design (CRD) with five extract treatments (0%, 1%, 2%, 3%,4%) and four repetitions. The parameters used were motility, viability, and membrane integrity. The data were analyzed with ANOVA and Duncan's test. Based on the results of the ANOVA test, the addition of green tea extract has a significant effect on spermatozoa quality of PO cattle in frozen storage ($P<0.005$). The best results were obtained by the addition of 3% extract that was able to maintain the quality of post-freezing spermatozoa with average motility, viability, and membrane integrity of 47.44±0.166, 55.14±0.449, and 53.72±0.599. The addition of 3% green tea extract into tris egg yolk can maintain motility, viability, and membrane integrity in spermatozoa of PO cattle inside frozen storage, by adding green tea extract to diluent, spermatozoa can be stored for a long time at low temperatures making it easier for people to carry out artificial insemination.

Keywords: Antioxidants; frozen storage;green tea extract; quality of PO bull spermatozoa

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INTRODUCTION

Meat contains various nutrients that are good for health, especially macronutrients and micronutrients (Li, 2017). The demand for beef has increased due to public awareness of the fulfillment of animal protein. National meat demand in 2021 is estimated to reach 685 thousand tons, while national beef production only rises to 425 thousand tons (Ministry of Agriculture, 2020). There will be an increase in demand for meat in 2024 of 3.36 kg/capita/year from the previous 2.72 kg/capita/year (Agus and Widi, 2018). The goverments in 2016 launched a programs aimed to increase the number of domestic cattle population by increasing pregnancy in cows. (Ministry of Agriculture, 2016). The government is targeting in 4 million productive females cows with successful pregnancy of 75% (Rusdiana and Soeharsono, 2017). The effective method used is artificial insemination, Artificial Insemination (AI) technology can be used as an alternative solution (Singh *et al*., 2016).

Artificial Insemination (AI) is one of reproductive technology alternative that has been widely applied by breeders around the world. AI is applied to the reproductive capacity and improve genetic quality at an affordable cost, uncomplicated process, and fast offspring production (Ren *et al*., 2019). AI technology is a program to introduce superior spermatozoa into the reproductive tract of female cattle with human assistance using an insemination gun (Suharyati and Hartono, 2013). The application of AI can increase the breeding capacity of males which allows higher quality selection rates by reducing the risk of spreading infectious diseases in livestock (Reda *et al*., 2015). The use of AI can improve genetic quality because the AI process is selected and only uses semen from males with superior characteristics (Santolaria, *et al*., 2015).

Sperm cryopreservation is an effective method that can be used to maintain male fertility in both human and animals (Sharma, 2011). Frozen semen are widely used in artificial insemination and can be stored for a long time (Yeste *et al.,* 2017). The quality of frozen semen is a crucial determinant of the success of AI (Casali *et al*., 2017). Frozen semen storage can maintain the motility and viability of spermatozoa in a continuous depository (Morrell *et al*., 2018). Freezing process

can cause a physical damage on spermatozoa, also inducing the reduction on percentage of live sperm (Kaka *et al.,* 2016; Ugur *et al.,* 2019). Spermatozoa damage is a result of Reactive Oxygen Species (ROS) that are produced during oxidative metabolic processes in the body (Sariözkan *et al*., 2014). The precense of ROS can affect sperm quality and could cause sperm be unable to fertilize (Len *et al.,* 2019). Damage caused by the freezing process can be prevented by choosing the right type of diluent. The diluent must be able to maintain pH, protect spermatozoa from cold shock, and contain nutrients that are needed by the spermatozoa to live. There are several types of diluents such as skimmed milk egg yolk, Andromed®, and tris egg yolk. Tris egg yolk is one of the commonly used diluents (Baharum *et al*., 2017). Tris diluent is a buffer that can maintain the pH of spermatozoa, contains glucose which is useful as an energy source for spermatozoa, and the egg yolk contains phospholipids and lecithin for protecting spermatozoa from cold shock during the freezing process (Perumal *et al*., 2013).

Antioxidants can hold down membrane damage due to free radical activity (Wahjuningsih *et al*., 2021). Antioxidants are added to the diluent to prevent damage to the plasma membrane caused by lipid peroxidation (LPO) (*Fang et al*., 2017). Based on research by Zhang et al., (2012), the addition of antioxidant in freezing semen can neutralize ROS and improve the sperm quality. Green tea can reduces the level of oxidative stress (Martin *et al.,* 2017). Green tea (*Camellia sinensis*) has an intense antioxidant activity, richer in antioxidants compared to other forms of tea (Prasanth *et al.,* 2019). Green tea mainly contains of chatecins (Wierzejska, 2014). Cathecins are the components in polyphenol its including two non-ester catechins, (−)-epicatechin (EC) and (−)-epigallocatechin (EGC), and two ester catechins, (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) (Miyoshi *et al., 2020).* EGCG possessing the most number of phenolic hydroxyl groups manifests the strongest antioxidant activity in catechins. (Zhao *et al.,2019).* According to Park and Yu (2017), the addition of green tea extract to semen during the freezing process was able to reduce ROS. And based on Khan *et al.,* (2017) The antioxidant content in green tea extract can suppress the peroxidation effect to inhibit membrane damage to the acrosome and maintain the viability of frozen semen. So far adding green tea extract into extenders can maintain the precentage of live sperm during cryopreservation. This study aimed to calculate the right concentration of green tea extract to be added to tris egg yolk during storage of Ongole Crossbreed (PO) semen on frozen storage assessed from motility, viability, and membrane integrity.

METHODS

Preparation of green tea extract

The steps taken in the making of green tea extract (*Camellia sinensis*) are to grind the dried extract using a mortar and macerate with 96% ethanol solvent. Maceration extract is placed in a closed container and stored at room temperature. The filtrate is evaporated with a rotary evaporator to make green tea extract paste (Priharyanthi, 2021).

Preparation of semen extender

The diluents of Tris Egg Yolk according to Arif (2022) are 3.03 grams of Tris aminomethane, 1.78 grams of citric acid, 1.25 grams of Fructose, and 0.1 grams of Penicillin and streptomycin. The diluents were put into an Erlenmeyer tube and dissolved in 100 ml of distilled water. Thereafter the solution is homogenized using 0.22 µm millipore. Sterilized eggs are broken and separated from the yolks and egg whites. The sterile tris solution was mixed with egg yolks in a ratio of 80 ml for tris and 20 ml of egg yolk and stored in the refrigerator right after. The diluent was divided into five treatments and each treatment was given the addition of green tea extract. The concentration of Green Tea extract for each treatment was 0% (Egg yolk Tris), 1% (Egg yolk Tris + 1% extract), 2% (Tris Egg yolk + 2 % extract), 3% (Tris egg yolk + 2 % extract), (tris egg yolk+3% extract) and 4% (Tris egg yolk+4% extract).

Semen collection

Storage of semen for PO bull is done by collecting semen using an artificial vagina at a temperature of 42℃ added with vaseline as a lubricant and an angler, and the female is used to increase the libido of males (Gohar, 2014). The obtained semen was then subjected to macroscopic and microscopic evaluation. The macroscopic evaluation includes observations of volume, color, odor, consistency, and pH. Microscopic evaluation includes mass motility, individual motility, membrane integrity, viability, and spermatozoa concentration (Arifiantini, 2012). Fresh semen that can be stored must have a

viability percentage of motility >70% and has a pH of 6.2-6.8 (Indriastuti et al., 2018).

Depository of frozen semen

The semen dilution is done in three-step. Diluents 1 and 2 were carried out at 37℃ in a water bath. Diluent 1 is added during the initial dilution process with a ratio of 1:1. The addition of the volume of diluent 2 is calculated using a predetermined formula. After that, the semen was stored in a cool tube at 5℃, followed by dilution 3. In diluent 3, 3.7% glycerol was added. Diluent 3 is carried out when the diluent is in the cooling tube and the spermatozoa are at 4-5℃.

The semen freezing process begins with lowering its temperature from 37℃ to 5℃ for the 2-hour equilibration process which is done in a cooling tube (Khan, 2017). After the filling sealing process continued, the straws are arranged on a rack and placed 1 cm above the surface of the liquid nitrogen to lower the temperature to -100℃. The straw was evaporated for 10 minutes and continued by inserting and storing the straws in a canister containing liquid nitrogen at -196℃ (Yendraliza, 2021).

Motility of spermatozoa

Motility observations before freezing and post-thawing were carried out by placing 20 µL of semen on an object-glass and observed with a light microscope using a magnification of 400x. Spermatozoa motility data were obtained by counting the moving spermatozoa from five different visual fields and calculated using a 0-100% scale (Prihantoko *et al*., 2020). Based on Santoso *et al.,* (2021) The characteristics of sperm motility can be divided into varoius movements, such as progressive motility, progressive fast motility, progressive slow motility, and immotile.

Viability of spermatozoa

Viability observations were done by adding eosin negrosin as a coloring agent to the semen on the object-glass. The smear preparations were dried using a hot plate and then observed using a light microscope with a magnification of 400x. The viability was projected from 200 spermatozoa in one field of view. If the colour agent can take up the stain and change the colour of spermatozoa into dark pink color, the spermatozoa considered dead. If the colour agent did not take up the stain, spermatozoa considered alive (Darussalam *et al, 2020*).

Membran Integrity of Spermatozoa

The addition of the Hypo Osmotic Swallen Test (HOST) solution to the semen needs to be done to observe the integrity of the spermatozoa membranes. 50 µL of semen was dissolved in 1 ml of HOST solution (HOST solution consisted of 7.35 g/L citric acid and 13.51 g/L fructose) (Zhang *et al*., 2021). The semen was dripped onto the object-glass and heated up on a hot plate at 37℃ for 45 minutes. The object-glass was then observed under a microscope using a magnification of 400x. Membrane integrity data was obtained by counting 200 spermatozoa in one field of view. Spermatozoa with straight tails were dead spermatozoa, while spermatozoa that were still alive were marked with a curved tail.

Data Analysis

The data from each treatment were analyzed by transforming the data into Arcsin which is then tested for normality using the Kolmogorof-Smirnov test. If the data is normally distributed, the one-way ANOVA test is continued and followed by Duncan's test to determine the difference in each treatment.

RESULTS AND DISCUSSION

Based on the results of semen observation, the volume of fresh semen was 9 ml, pH 6.5, mass motility 3+, and individual motility of 80%. These results indicate that fresh semen can be processed into frozen semen because it has a motility value above 70%. According to Centola (2018), PO bull semen is considered good when it has around 70-90% range of motility.

Motility is one of the common parameters used in semen evaluation (Moradpour, 2019). Observation of spermatozoa motility was done by dripping 20 µL of semen on an object-glass which was then covered with a cover glass and observed using a light microscope using a magnification of 400x (Prihantoko *et al*., 2020).

Table 1. Average Motility Percentage and Standard Deviation

Treatments	Average ± Standar Deviation of Motility (%)	
	Before Freezing	After Freezing
Tris + Green tea extract 0%	51.28±0.440[a]	34.83±0.522[a]
Tris + Green tea extract 1%	53.73±0.347[b]	40.11±0.531[b]
Tris + Green tea extract 2%	56.95±0.405[c]	42.06±0.276[c]
Tris + Green tea extract 3%	60.84±0.701[e]	47.44±0.166[e]
Tris + Green tea extract 4%	58.94±0.311[d]	44.50±0.917[d]

Notes: Different notations (a, b, c, d, e) on the same line indicate a significant effect on the results in each treatment. a = the lowest percentage notation, e = the best percentage notation.

ANOVA test showed that green tea extract in tris egg yolk has a significant effect in maintaining motility value (P<0.005). Based on table 1. the average value of the motility before freezing in the treatment group with green tea extract had a higher value than the control group. The highest average motility value was obtained by treatment with 3% green tea extract, which was 60.84±0.701 before freezing and 47.44±0.166 after freezing. The lowest motility value was acquired by control group with 0% green tea extract before freezing at 51.28 ± 0.440 and after freezing at 34.83 ± 0.522. Based on the Indonesian National Standard Agency (2017), frozen semen with a motility value of 40% is semen that meets the standard and can be distributed, which means that tris egg yolk with green tea extract meets the value of the National Standard.

Fresh semen freezing causes a decrease in motility value that reaches 80% before freezing. The reduction in motility value began with a change in temperature from 37℃ to 5℃ which leads to cold shock due to structural and functional changes in the lipid membrane of spermatozoa (Ducha *et al*., 2017). Decreased motility of spermatozoa can also be caused by the accumulation of excess Reactive Oxygen Species (ROS) during the freezing process. ROS result in changes in the lipid composition of the membrane (Dutta *et al*., 2019). According to Sariözkan et al. (2014), decreased motility in post-thawing is due to damage during the freezing process of semen caused by free radicals or ROS produced during oxidative metabolic processes in the body. Excessive ROS production also leads to damage in spermatozoa DNA which causes a decrease in fertility value (Thompson *et al*., 2014) A disrupted ion pump due to membrane damage leads to decreased sperm motility and impaired spermatozoa motility (Guthrie, 2012). High levels of ROS can cause damage to axonemal proteins and mitochondria, resulting in motility reduction and death in spermatozoa (Peris-Frau *et al*., 2020). The addition of antioxidants in the diluent is needed to prevent the accumulation of free radicals that can reduce the value of spermatozoa motility. Antioxidants are compounds that are given to suppress membrane damage due to free radical activity (Wahjuningsih *et al*., 2021).

Based on the results obtained, the group with the addition of green tea extract was more able to maintain motility from free radical attacks that could trigger ROS since green tea leaves (*Camelia sinensis*) have high antioxidant activity. Green tea mainly contains of chatecins (Wierzejska, 2014). Catechins in green tea act as chelating agents and can catalyze lipid membranes to prevent the occurrence of OS (Roychoundhury *et al*., 2017). Besides of catechin in green tea there is also has another potential antioxidants agent it is a vitamin E. Based on numerous research vitamin E be a supporting role for keeping the quality and quantity of sperm, fertilization, and fertility in humans (Mahmoudi *et al.*, 2018). According to Kročková *et al*. 2021, the antioxidant content in green tea extract can maintain the motility of spermatozoa from lipid peroxidation, thereby increasing the motility of spermatozoa. Following results of the studies where the Tris treatment, which was not given the addition of Green Tea extract had the lowest average motility value among other treatments, which was only 34.83±0.522 in post thawing.

Antioxidants can be utilized as lipid peroxidation inhibitor reactions that can damage spermatozoa membranes and reduce spermatozoa motility during the frozen storage process. According to Park and Yu (2017), the addition of green tea extract to semen during the freezing process was able to reduce ROS. Antioxidants were added to extenders can protect spermatozoa from oxidative stress caused by ROS during frozen storage (Allai *et al., 2018*). Giving an enormous amount of antioxidants can have a terrible impact because it leads to a loss of its effectiveness as an antioxidant and even causes peroxides (Wahjuningsih *et al*., 2021)

This research was accomplished by adding

eosin negrosin to the semen that had been placed on an object-glass and making smear preparations (Bintara *et al*., 2022). This method is used to analyze the viability and evaluate the morphological structure of spermatozoa (Kondracki *et al*., 2017).Observations were done by color changes that appear on the spermatozoa if the coloring agent can penetrate the spermatozoa and turn them into dark pink color, and if the dye is not able to penetrate the spermatozoa so that the spermatozoa are not stained, the spermatozoa are still alive (Fischer *et al*., 2020).

Table 2. Average Viability Percentage and Standard Deviation

Treatments	Average ± Standar Deviation of Viability (%)	
	Before Freezing	After Freezing
Tris + Green tea extract 0%	57.08±0.601[a]	39.06 ±0.607[a]
Tris + Green tea extract 1%	60.16±0.771[b]	43.11±0.495[b]
Tris + Green tea extract 2%	62.02±1.151[c]	45.31±0.830[c]
Tris + Green tea extract 3%	69.69±0.653[e]	55.14±0.449[e]
Tris + Green tea extract 4%	64.06±1.115[d]	48.04±1.131[d]

Notes: Different notations (a, b, c, d, e) on the same line indicate a significant effect on the results in each treatment. a = the lowest percentage notation, e = the best percentage notation

The green tea extract in egg yolk tris has shown a significant effect in maintaining the viability value (P<0.005) by ANOVA Test. Based on table 2. the average value of the viability before freezing in the treatment group that has the addition of green tea extract had a higher value. The highest viability value was acquired by treatment with 3% green tea extract, which was 69.69 ± 0.653 before freezing and 55.14 ± 0.449 after freezing. The lowest viability value was owned by tris treatment as a control with a value before freezing at 57.08 ± 0.601 and after freezing at 39.06 ± 0.607.

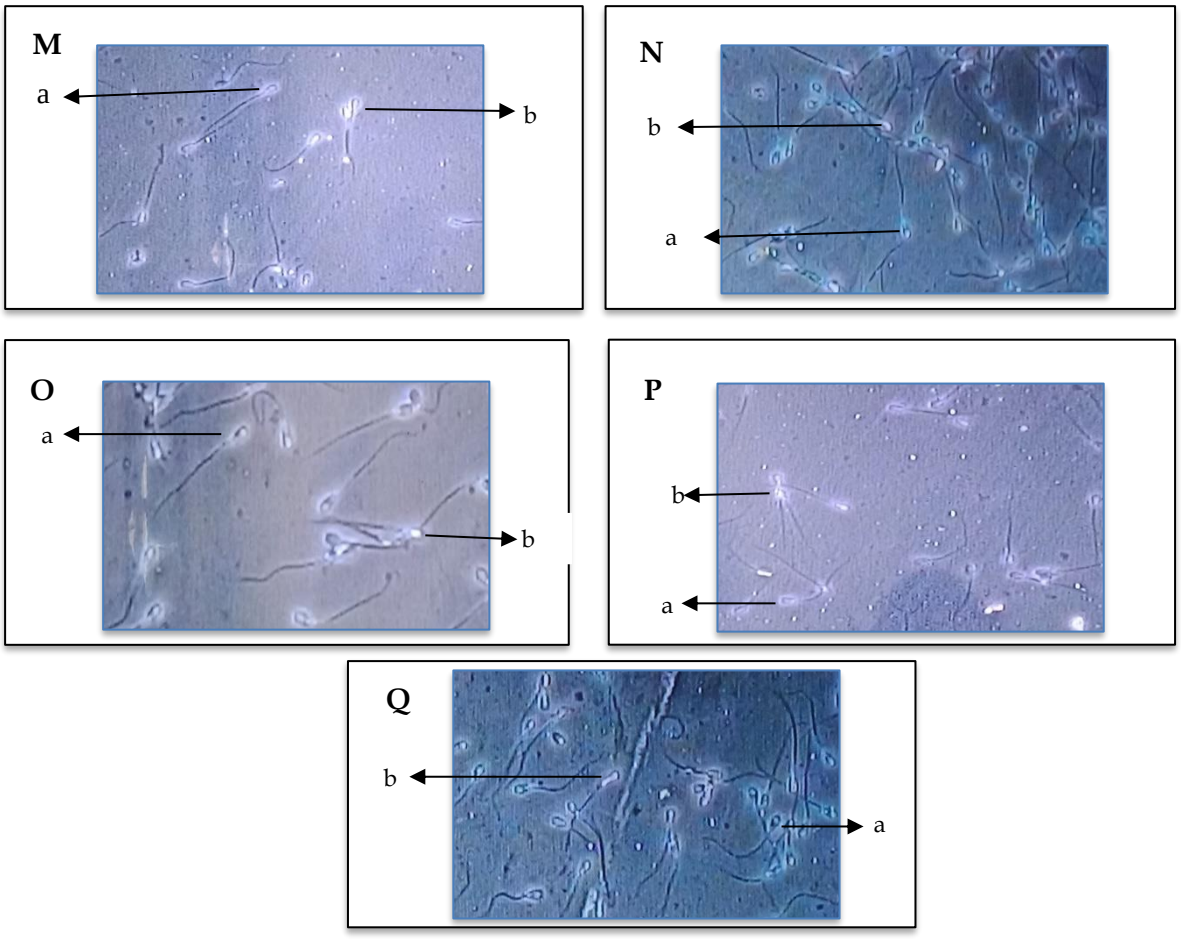

Figure 1. Viability of PO bull spermatozoa

Description : M = Tris Egg Yolk, N = Tris Egg Yolk + Green Tea Extract 1%, O = Tris Egg Yolk + 2% Green Tea Extract, P = Tris Egg Yolk + 3% Green Tea Extract, Q = Tris Egg Yolk + Green Tea Extract 4%, Notation on the picture Shows (a) live spermatozoa, (b) dead spermatozoa.

Figure 1 shows that the spermatozoa stained by an eosin-negrosin coloring agent are used to determine the viability of the spermatozoa. Spermatozoa that are still alive are indicated by the notation (a) where the head of the spermatozoa is dark and colorless, whereas purplish colors are specified for the dead spermatozoa (b).

According to the test result, adding up to 3 percent green tea leaves extract in tris egg yolk can provide the highest viability value. The addition of green tea extract has a positive influence on the survival of spermatozoa. The antioxidant content in green tea extract can suppress the peroxidation effect to inhibit membrane damage to the acrosome and maintain the viability of frozen semen (Khan *et al*., 2017).

Sperm plasma membrane integrity was the essential criteria for evaluating fertilization ability that directly decreased depending on sperm lipid peroxidation levels (Lee *et al*., 2017). The integrity of the membrane is an indicator of the membrane mechanism in regulating the traffic in and out of substances and ions in the process of cell metabolism. According to Gohar *et al*. (2014), the integrity of spermatozoa membranes was tested using the Hypo Osmotic Swallen Test (HOST) method.

Table 3. Average Percentage of Membrane Integrity and Standard Deviation

Treatments	Average ± Standar Deviation of Mmbrane Integrity (%)	
	Before Freezing	After Freezing
Tris + Green tea extract 0%	56.20±0.622[a]	40.32 ±0.829[a]
Tris + Green tea extract 1%	59.03±0.658[b]	43.65±0.261[b]
Tris + Green tea extract 2%	61.37±0.672[c]	46.13±0.288[c]
Tris + Green tea extract 3%	67.42±1.292[e]	53.72±0.599[e]
Tris + Green tea extract 4%	61.69±0.179[d]	49.88±0.889[d]

Notes: Different notations (a, b, c, d, e) on the same line indicate a significant effect on the results in each treatment. a = the lowest percentage notation, e = the best percentage notation.

The results of the ANOVA test showed that the addition of green tea extract into egg yolk tris shows a significant effect in maintaining the membrane integrity value (P<0.005). Based on table 3. the average value of membrane integrity's percentage before freezing in the treatment group that had green tea extract had a higher value. The highest average value of membrane integrity was obtained by treatment with 3% green tea extract, which was 67.42±1.292 before freezing and 53.72±0.599 after freezing. The tris treatment has the lowest membrane integrity value before freezing at 56.20 ± 0.622 and after freezing at 40.32 ± 0.829.

The highest value of membrane integrity was acquired by the 3 percent extract treatment both before freezing and post thawing. This is the opinion of Kang *et al*. (2020), who states that there is a relationship between motility, viability, and membrane integrity. According to Gangwar *et al*., (2018), when live spermatozoa are given a HOST (Hypo Osmotic Swelling Test) solution, it will make the tail cells of the spermatozoa swell and curl since cells are trying to balance the fluid inside and outside the cell where the hypoosmotic solution will enter into the membrane. Tris Egg Yolk are commonly used in bull semen both in frozen storage and refrigerator storage. The addition of green tea extract to tris egg yolk diluent was able to increase the viability of PO bull spermatozoa during the frozen storage process. Tea leaves contain secondary metabolites, namely antioxidants, which are able to fight the formation of free radicals in spermatozoa during the freezing process, by adding green tea extract to diluent, spermatozoa can be stored for a long time at low temperatures and making it easier for people to carry out artificial insemination.

CONCLUSION

The study concluded that the addition of 3% green tea extract into tris egg yolk is the right concentration that can maintains the motility value after freezing at 47.44±0.166, viability value at 55.14±0.449, and membrane integrity values by 53.72±0.599. For further studies, green tea extract can be added into various diluents to increase the

motility of spermatozoa during frozen storage or frozen storage process process.

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