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The effect of addition of *Allium cepa* extract in diluent Ringer's-Dextrose on *Gallus domesticus* sperm quality at 5°C

Pengaruh penambahan ekstrak bawang merah dalam pengencer Ringer's Dextrose terhadap kualitas sperma Gallus domesticus pada suhu 5°C

Sakinato Mazidda[1], Suyadi[2*], Dyah Hikmawati[3], Alditya Putri Yulinarsari[4]

[1]Department of Biology, Faculty of Science and Technology, Airlangga University, Mulyorejo, Surabaya, 60115, Indonesia
[2]Department of Animal Production, Faculty of Animal Science, Brawijaya University, Lowokwaru, Malang, 65145, Indonesia
[3]Department of Physics, Faculty of Science and Technology, Airlangga University, Mulyorejo, Surabaya, 60115, Indonesia
[4]Department of Animal Husbandary, Politeknik Negeri Jember, Jl. Mastrip Po Box 164 Jember, 68101, Indonesia
*Corresponding author: suyadi@ub.ac.id

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ABSTRACT

Gallus domesticus is a local chicken of Indonesia which should be preserved as a local genetic resource because it has the advantage of high adaptability environment and has an economic value. This study aimed to determine the quality of *Gallus domesticus* semen the effect of storage time by the addition of *Allium cepa* extract (ACE) in the Ringer's Dextrose diluent at temperatur of 5°C. The making of Allium cepa extract was done by using the maceration and *Gallus domesticus* semen was collected using the female teaser method. The collected semen was divided into 4 treatments, T0 (Ringer's Dextrose without ACE); T1 (Ringer's Dextrose + 0.02 ml ACE); T2 (Ringer's Dextrose + 0.04 ml of ACE); T3 (Ringer's Dextrose + 0.06 ml of ACE) and evaluation of the semen quality including individual motility, viability, and abnormality was observed within 6, 8, and 24 hours. The experimental design used a Completely Randomized Design, which proceeded with the Duncan Multiple Range Test. The analysis significant differences ($P<0.01$) for individual motility and ($P<0.05$) for sperm viability. The best results were shown by T2 treatment. Conclusion of study the addition of *Allium cepa* extract antioxidant in Ringer's dextrose diluent can maintain the quality of spermatozoa *Gallus domesticus* during storage temperature at 5°C so it is eligible for artificial insemination.

Kata kunci:
Allium cepa
Artificial insemination
Gallus domesticus

ABSTRAK

Gallus domesticus merupakan ayam lokal Indonesia yang harus dilestarikan sebagai sumber daya genetik lokal karena memiliki keunggulan daya adaptasi yang tinggi terhadap lingkungan dan memiliki nilai ekonomis. Tujuan penelitian ini untuk mengetahui kualitas semen Gallus domesticus terhadap pengaruh lama penyimpanan dengan penambahan ekstrak Allium cepa pada pengencer Ringer's Dextrose suhu 5°C. Pembuatan ekstrak Allium cepa dilakukan dengan cara maserasi dan semen Gallus domesticus dikumpulkan dengan metode teaser female. Semen yang terkumpul dibagi menjadi 4 perlakuan yaitu T0 (Ringer's Dextrose tanpa ekstrak Allium cepa); T1 (Ringer's Dextrose + 0,02 ml ekstrak Allium cepa); T2 (Ringer's Dextrose + 0,04 ml ekstrak Allium cepa); T3 (Ringer's Dextrose + 0,06 ml ekstrak Allium cepa) dan evaluasi kualitas semen meliputi motilitas individu, viabilitas, dan abnormalitas diamati dalam waktu 6, 8, dan 24 jam. Rancangan percobaan menggunakan Rancangan Acak Lengkap yang dilanjutkan dengan Uji Jarak Berganda Duncan. Analisis menunjukkan perbedaan yang sangat signifikan (P<0,01) untuk motilitas individu dan (P<0,05) untuk viabilitas sperma. Hasil terbaik ditunjukkan pada perlakuan T2 yaitu Ringer's Dextrose + 0,04 ml ekstrak Allium cepa. Kesimpulan penelitian ini penambahan antioksidan ekstrak Allium cepa pada pengencer Ringer's dextrose yang mampu mempertahankan kualitas spermatozoa Gallus domesticus selama penyimpanan suhu 5°C dan perlakuan T2 layak untuk dilakukan inseminasi buatan.

INTRODUCTION

Chicken products (such as eggs and meat) is Indonesian's staple foods. *Gallus domesticus* has an economic value due to its distinctive taste of meat and egg. Therefore, Indonesians put so much interest and have high demand towards it (Rochmi & Sofyan, 2019). Some advantages of the *Gallus domesticus* breeding compared to purebreed chicken are including the fact that it is more resistance to the disease, has high adaptability to the environment, as well as its breeding is relatively easier. However, *Gallus domesticus* has some disadvantages, which are the slow growth rate and low production ability because of the slow genital development, in which it must goes through the process of incubating (Andayani, Setyawati, & Joni, 2018). On the top of that, the period of laying eggs is quite long due to the necessity to nurture the chicks.

The data from the Central Statistics Agency, Indonesia reported that in 2019, the production for chicken more of less three million per ton for a year (Badan Pusat Statistika, 2020). This showed the importance for increasing the chicken production, one of which is the species of *Gallus domesticus*. Some methods were made to increase the chicken production. Artificial Insemination (AI) is an effective way to overcome this obstacle. Storage parameters such as temperature, diluent, and energy sources for spermatozoa form the most important parameters for the success of AI (Kharayat et al., 2016). The success factor of chicken AI was strongly influenced by semen quality (Iswati, Isnaini, & Susilawati, 2018). In her research, (Iswati et al., 2018) succeeded in collecting the semen by using the t*easer female* method with an average semen volume of 0.5 ± 0.029, which was greater than the massage method conducted by Usman, Sir, & Sani (2019), which had an average volume of 0.21 ml.

Another factor that influenced the quality of semen was the semen dilution material which was used just before the insemination. The storage of semen at the temperature of 4 ^{o}C using Ringers diluent was able to maintain the quality of semen for 8 hours in maximum because Ringers' solution contains various mineral salt which has buffer and isotonic features to support the sperm motility for a longer period (Danang, Isnaini, & Trisunuwati, 2012). The use of lactate ringers diluent was able to maintain semen quality for 32 hours in cold temperatures (Iswati et al., 2018). The chicken's spermatozoa could be stored effectively for 7 days at 5^{o}C (Rochmi & Sofyan, 2019). The temperature and storage time of semen in the diluent affected the quality of the semen and its motility. At cooler temperature, the metabolism of semen would be slower because of its lower mobility. At warmer temperature, the semen would require more nutrients and the semen's nutrient storage time would be lesser, which then resulting the decreasing of motility.

As an effort to maintain the individual motility, sperm viability, and abnormality as a requirement to perform insemination could be done by storing semen samples at a temperature of 5^{o}C, aiming that sperm able to live longer so that the distribution of semen which would be inseminated to distant places could be realized. The long storage period of semen could reduce the quality of sperm. Thus, to overcame such an issue, it was needed to add the red onion extract as an antioxidant so that the sperm quality could be maintained, stated that onion (*Allium cepa* L.) and quercetin protect against oxidative damage and have positive effects on multiple functional parameters of spermatozoa, including viability and motility (Chae et al., 2017).

Problems that emerged in the process of cold storage of semen was the damage of plasma membrane due to the formation of peroxidative lipid (Usman et al., 2019). It was known that the contact between semen and excessive oxygen caused the peroxidative damage. The damage was caused by the sperm membrane which contained more unsaturated fat which is very susceptible to the damage caused by free radical peroxidation. The attempt to maintain the quality and viability of sperm from the damage caused by free radicals could be minimized by the addition of antioxidants compounds. *Allium cepa* ameliorated CdSO(4)-induced alteration in testicular weight, sperm count, sperm motility, and sperm morphology (Ige et al., 2012). It also showed that *Allium cepa* attenuated the derangement of lipid peroxidation profile in testicular tissues caused by CdSO(4) exposure.

Semen storage that could be conducted was by using cold storage at the temperature of 3-5°C. This was intended to inhibit the sperm activity both physically and chemically. The sperm quality during storage time must be evaluated to determine how viable and fertile spermatozoa

in the female reproductive system. By knowing the best storage time, the quality of semen could be maintained and the male could be taken its advantage more efficient (Iswati et al., 2018).

The novelty from this research find the alternative materials as a source of hormones and easily obtained. The previous research showed improvement of sperm quality by providing energy level in ration (Haryuni, Lidyawati, Khopsoh, & Hasanah, 2020). This study aimed to determine the quality of *Gallus domesticus* semen the effect of storage time by the addition of *Allium cepa* extract (ACE) in the Ringer's Dextrose diluent at temperatur of 5°C.

MATERIALS AND METHODS

Location

The *Gallus domesticus* semen storage was performed at the Poultry Installation and the semen test was conducted at the Indonesia Animal and Reproductive Health Laboratory, Malang Agricultural Development Polytechnic.

Material Research

The red onion (*Allium cepa*) obtained from the local market was extracted by maceration method, using 50% ethanol solvent, and evaporated with a vacuum rotary evaporator. The semen from four *Gallus domesticus* that were 12 months old and weighed 2.5 kg was obtained from the Poultry Installation, Malang Agricultural Development Polytechnic. The collection of fresh *Gallus domesticus* semen was conducted using the female teaser method. The diluent used for the semen dilution was Ringer's Dextrose (10% infusion reg no.: GKL9230500249B1) with Dextrose, $C_6H_{12}O_6$, H_2O: 50.0 g and water for injection add: 500ml).

Semen Test and Dilution

Individual motility of spermatozoa was observed using a microscope with 400x magnification and a glass cover. Assessment of individual motility can be seen by how many spermatozoa that move progressively forward compared to spermatozoa that stay stationary. The calculation of the viability percentage was using smeared preparations with eosin-nigrosine staining (Chae et al., 2017).

Viability calculation formula (1)

$$= \frac{\text{total live sperm}}{\text{total live sperm + total dead sperm}} \times 100\%$$

Preparations that had been used to calculate the viability value could be used to determine the percentage of spermatozoa abnormality. Abnormal spermatozoa were characterized by a broken or circular tail and a small or double head (Kondracki, Wysokinska, Kania, & Górski, 2017).

Abnormal calculation formula (2)

$$= \frac{\text{total abnormal sperm}}{\text{total abnormal sperm + total normal sperm}} \times 100\%$$

The number of spermatozoa per milliliter of semen was calculated using a hemocytometer. The concentration test was carried out by absorbing the semen with an erythrocyte pipette up to number 0.05 and then adding it with hypertonic liquid (NaCl) up to number 1. The solution was mixed into a homogeneous mixture by 8-forming shaking for 3 minutes. Two to three drops of mixed semen were discarded because they only contain hypertonic fluid. Then, the solution was being homogenized again for one minute and another drop was removed. The solution was dropped on a hemocytometer and closed with a glass cover. The calculation was carried out on a microscope with 400x magnification in five counting chambers and compiled in the unit of millionper ml (Susilawati, 2013).

Maintenance and semen collection of indigenous chicken with female method performed at the College of Agricultural Extension. Semen obtained from collection was divided into 4 treatments (Mazidda, Suyadi, & Hikmawati, 2020). The collected semen was divided into 4 treatments, specifically T0 (Ringer's Dextrose without *Allium cepa* extract), T1 (Ringer's Dextrose + 0.02 mL of *Allium cepa* extract), T2 (Ringer's Dextrose + 0.04 mL of *Allium cepa* extract) and T3 (Ringer's Dextrose + 0.06 mL of *Allium cepa* extract). The evaluation of the semen quality during storage time at the 5ºC was observed at 6, 8 and 24 hours, The experimental design used a Completely Randomized Design (CRD), the data analysis used Analysis of Variance (ANOVA), and proceeded with the Duncan Multiple Range Test (DMRT).

RESULT AND DISCUSSION

Result of Stroage of Semen *Gallus domesticus*

The *Gallus domesticus* semen which was collected using the *female teaser* method was examined macroscopically which included

volume, color, pH, and consistency. Moreover, the microscopic characteristics were also examined, those were including the mass motility, individual motility, concentration, viability, and abnormality. The results of semen quality test in this study could be seen in Table 1.

Table 1 shown that the quality of semen

Table 1. Test results of *Gallus domesticus* fresh semen quality (n=6)

Variable	Average
Volume (ml/ejaculation)	0.5 ± 0.06
Consistency	Thick
pH	7.3
Color	Milky white
Smell	Odor of chicken
Mass Motility	3+
Individual Motility (%)	85
Viability (%)	94.17 ± 1.47
Abnormality (%)	5.16 ± 0.75
Concentration (million/ml)	354.50 ± 33.72

obtained from 6 replications was in normal condition. This was in accordance with the opinion of which was used as a standard for data comparison in the table above. The volume of semen in this study was still within the normal range, in which the standard volume of chicken's semen ranged from 0.2-0.5 mL (Iswati et al., 2018). The volume resulted by the *teaser female* semen storage method in this study was greater than that of Shanmugam, Vinoth, Rajaravindra, & Rajkumar (2014), which was 0.29 mL, Sun et al., (2019) with 0.29 mL and Usman et al., (2019) with 0.21 mL, who collected the semen using *massage* method.

The semen collection using the *teaser female* method resulted a higher volume compared to the massage method. The volume of semen produced is influenced by several factors internal and external factors. Internal factors were including hormones, metabolism, heredity, food, age, and general health of the male (Okoro, Mbajiorgu, & Mbajiorgu, 2016). While external factors were the atmosphere, the shelter, management, the shelter, and the weather (Madeddu et al., 2009). In the biological term, the semen collection using *teaser female* method made the livestock naturally had a high libido level due to the stimulation of a *teaser female* chicken so that semen ejaculation could be optimal. This was because the ejaculation was preceded by a perfect erection process and occurred on its own, without any coercion. Furthermore, the possibility for the occurrence of damage to the male reproductive system was very small, so that the male chicken could be taken the advantage for longer period of time, which was in contrast to the massage method. The massage method made the livestock to be unnaturally stimulated. Thus, the ejaculated semen would not be optimal and the ability of male chicken to be taken semen could not last for long. The continuous nerve stimulation would reduce the value of the reproductive nerve threshold which then caused the male chicken difficult to be stimulated for collecting its semen. On the top of that, the color of semen in this study was milky white and considered normal as reported by (Usman et al., 2019).

The value of pH examination using litmus paper showed a pH result of 7.3. This result was in accordance with that the pH of chicken semen was between 7.2-7.6 (Iswati et al., 2018). Massa motility was obtained 3+ which was indicated that it was in accordance with Rochmi & Sofyan (2019) who stated that the excellent mass motility was 3+ and marked by cloud-like huddling sperm. Individual motility and viability produced were greater at 85% and 94.17 ± 1.47% compared to Sun et al., (2019) at 67.84%, 71.17% and (Shanmugam et al., 2014) at 46.25 %, 93.01 ± 2.39%.

Motility of Spermatozoa

Table 2 shown the control treatment (T0) could maintain the motility up to 8 hours at 5^{o}C with motility average of 41.67 ± 2.58%$^{a.}$ This result was not different from T1 but was significant different with T2 and T3. The T2 and T3 treatments were able to maintain motility for up to 24 hours with the motility percentage T2 of 43.49 ± 2.74%^{b} and T3 40.00 ± 4.47%^{b} compared to the T0 and T1 treatments, *Allium cepa* extract contains the antioxidant compound quercetin which functions to protect sperm cells from free radical damage.

The decrease in individual motility during storage is thought to be due to the influence of toxic substances caused by dead spermatozoa on living spermatozoa or from diluents that have been oxidized due to storage can cause free radical compound damage which can damage the

Table 2. The average motility of Gallus domesticus sperm with different concentrations of *Allium cepa* extract in Ringer's Dextrose diluent stored at 5°C (%) (n=6).

Group of treatments (ml)	Observation time (h)		
	6	8	24
T0 (0)	52.50±2.74[a]	41.67±2.58[a]	21.67±2.58[a]
T1 (0.02)	55.83±3.76[a]	44.17±3.76[a]	27.50±2..74[a]
T2 (0.04)	62.50±2.74[b]	52.50±2.74[b]	43.49±2.74[b]
T3 (0.06)	60.00±3.16[b]	50.00±3.16[b]	40.00±4.47[b]

[abc]Different superscripts in the same row showed significant differences (P<0.01)

integrity of the spermatozoa plasma membrane and have a negative impact on metabolism resulting in spermatozoa death (Khaki, Khaki, & Rajabzadeh, 2017). Sperm motility of T2 and T3 which was stored for 24 hours at 5°C could still be inseminated compared research result (Mazidda et al., 2020) semen that stored at room temperature was only able to maintain motility for ≥40% at 4 hours of storage. Semen which was stored at a temperature of 5°C experienced limited motion and delayed metabolic activity. (Bonato, Cornwallis, Malecki, Rybnik-Trzaskowska, & Cloete, 2012) stated that the higher temperature of the semen storage, the higher the movement of spermatozoa would be, the movement of sperm required energy while the formation of energy produced by spermatozoa was limited. The addition of 300 mg red onion extract to the rat semen had a sperm motility average of 66.66 ± 0.76%[a] within 12 hours of storage temperature at 4-5°C (Lukman, Busono, Wahyuningsih, & Suyadi, 2014). The quercetin flavonoid compound in red onion (*Allium cepa*) has antioxidant and was able to maintain the sperm motility quality of rats exposed to cadmium and trigger lipid peroxidation, which leads to oxidative stress (Ige et al., 2012).

Figure 1. shown that the longer of the semen storage time decrease more, this happen due to the fact that the process of adaptation of spermatozoa with the process of dilution could affect the activity of metabolism and availability of the nutrients. The cooling process caused decrease in sperm cell motility because the sperm got experiences a cold shock (Lukman et al., 2014). Oxidative stress which also occurred during the cold storage could cause the decreasing of motility and the increasing of the dead sperm

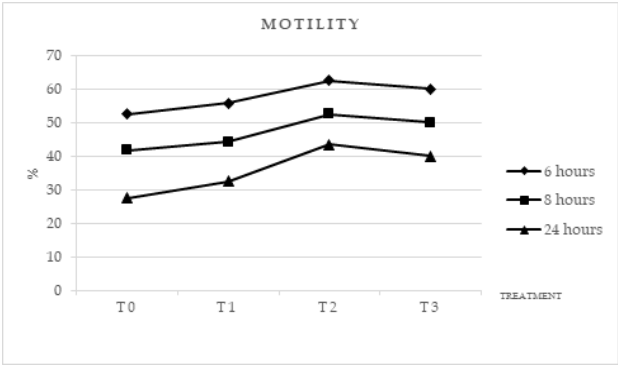

Figure 1. Percentage of sperm motility at storage temperature of 5°C for 6, 8 and 24 hours.

cell (Khan, 2011).

Viability of Spermatozoa

The results ANOVA test shown the treatment of the additional different concentration of *Allium cepa* extract in Ringer's Dextrose diluents, for 24 hours storage at the temperature of 5ºC gave a significant difference (*P*<0.05) to the percentage of spermatozoa viability (Table 3). The highest average viability during 24 hours of storage (T2), specifically the 6th hour (83.26 ± 1.59%[c]), the 8th

Table 3. The average viability of *Gallus domesticus* sperm with different concentration of *Allium cepa* extract in Ringer's Dextrose diluent at 5ºC (%) (n=6)

Group of treatments (ml)	Observation time (h)		
	6	8	24
T0 (0)	75.08±2.39[a]	67.71±2.42[a]	58.23±1.88[a]
T1 (0.02)	73.28±1.21[a]	68.71±1.37[a]	58.05±2.31[a]
T2 (0.04)	83.26±1.59[c]	77.26±2.83[c]	67.98±2.05[c]
T3 (0.06)	79.67±1.49[b]	73.32±3.70[b]	62.41±1.08[a]

[abc]Different superscripts in the same row showed significant differences (P<0.05)

hour (77.26 ± 2.83%[c]) and the 24th hour (67.98 ± 2.05%[c]) compared to T0, T1, and T3.

The addition of 0.04 mL and 0.06 mL *Allium cepa* extract in Ringer's Dextrose diluent was more effective in maintaining the viability compared to T0 and T1 treatments during 24 hours of storage at the temperature of 5ºC. At 24 hours storage with the temperature of 5ºC, T0 (Ringer's Dextrose without *Allium cepa* extract) was able to maintain the viability for ≥40% namely 58.23 ± 1.88%[a] but could not maintain motility for ≥40%, compared result research (Mazidda et al., 2020) at room temperature storage which was only able to maintain the viability for ≥40% within 4 hours

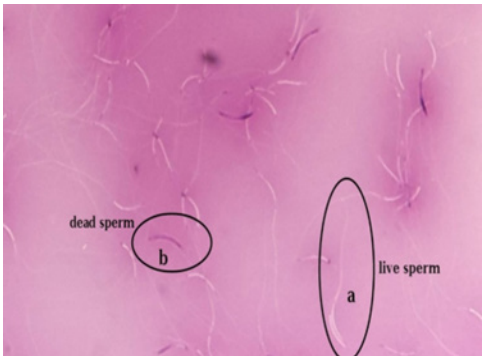

Figure 2. The viability of *Gallus domesticus* sperm by eosin-nigrosine staining (a) live sperm; (b) dead sperm.

storage. The effect of temperature and duration of storage of cement affected the decreasing of motility but did not affect its viability (Bonato et al., 2012). Meanwhile, changes in pH caused a decrease in viability. The addition of 300 mg of red onion extract to rat semen had sperm motility average of 82.5 ± 1.50%[ab] for 12 hours storage with a temperature of 4-5°C (Asadpour, Azari, Hejazi, Tayefi, & Zaboli, 2013).

In Figure 2. shown dead sperm cells were able to absorb the eosin-nigrosine staining while live sperm cells could not to absorb the eosin-nigrosine (Kondracki et al., 2017) stated that sperm cells did not absorb the staining because their plasma membranes were fully reliable, meanwhile, the dead sperm cells' plasma membranes have been damaged so that they were able to absorb the stain. (Chen et al., 2019) reported that the evaluation of spermatozoa viability using the eosin-nigrosine staining test was recommended by the fifth edition of the World Health Organization (WHO) Laboratory Manual for examining human semen. By using this staining method, the immotile sperm could be known.

Abnormality of Spermatozoa

After during 24 hours of storage, T2 treatment shown the lowest average abnormality which were 11.14 ± 0.46[a], 12.79 ± 0.49[a], 16.59 ± 0.65[a], compared with T0, T1, and T3 (Table 4). For 24 hours in storage with the temperature of 5ºC, T2 and T3 semen were still feasible to be inseminated because it had an abnormality value below 20%, and the percentage of motility, viability was ≥40%. The high spermatozoa abnormality would affect the fertilization (Iswati et al., 2018). Thus, low abnormality was

Table 4. The average abnormality of *Gallus domesticus* sperm with different concentrations of *Allium cepa* extract in Ringer's Dextrose diluent at 5ºC (%) (n=6)

Group of treatments (ml)	Observation time (h)		
	6	8	24
T0 (0)	14.01±0.43[c]	15.72±0.55[c]	18.53±1.33[b]
T1 (0.02)	14.02±1.20[c]	15.36±0.93[b]	17.70±0.99[a]
T2 (0.04)	11.14±0.46[a]	12.79±0.49[a]	16.59±0.65[a]
T3 (0.06)	12.64±0.65[b]	14.49±1.27[b]	17.77±0.80[a]

[abc]Different superscripts in the same row showed significant differences (P<0.05)

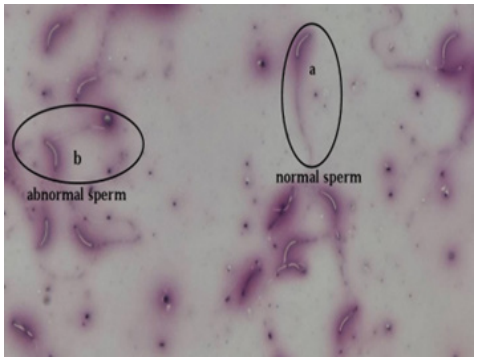

Figure 3. The abnormality of *Gallus domesticus* sperm. (a) normal sperm; (b) abnormal sperm.

an artificial insemination requirement and the factor that affect abnormality itself such as age, temperature, maintenance management, dilution, and environment. The addition of 5 mL red onion extract to rat semen was able to maintain the sperm quality during cold storage (Jeje, Adegbite, Akindele, Kunle-Alabi, & Raji, 2020).

The study results in Figure 3. shown abnormalities of the wrapped around and broken tail. (Iswati et al., 2018) mentioned that abnormality was classified into 2 types, primary and secondary abnormality. The primary abnormality occurred during the process of spermatogenesis that associated with the head and acrosomes, while the secondary abnormality occurred after the process of spermatogenesis, specifically when the ejaculations, a place to inhabit, semen evaluation, and semen processing. Generally, primary abnormality include deformity in the head (too big or small, more than one head). Secondary abnormality could be characterized by a broken tail, wrapped and bent tail. The head abnormality occurred during spermatogenesis in the seminiferous tubules whereas the tail

abnormalities occurred) due to dilution process and environmental factors, including storage period (Danang et al., 2012).

CONCLUSION

In conclusion, the addition of *Allium cepa* extract antioxidant in Ringer's dextrose diluent is able to maintain the quality of spermatozoa *Gallus domesticus* during storage temperature at 5°C. The addition of 0.04 ml extract *Allium cepa* in Ringer's dextrose diluent is an appropriate concentration in maintaining the quality spermatozoa of *Gallus domesticus* until the 24th hour either from the side of individual motility, viability, and spermatozoa abnormalities so that it is eligible for artificial insemination. It is recommended to do further research to findout fertility and hens' power of *Gallus domesticus* egg that isinseminated by semen diluted with Ringer's dextrose +0.04 ml *Allium cepa* extract temperature at 5°C save up to 24 hours.

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