Eco. Env. & Cons. 26 (November Suppl. Issue) : 2020; pp. (S49-S54)
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ISSN 0971–765X

Effect of addition of onion (*Allium cepa* L.) extract in ringer's diluent on spermatozoa quality of *Gallus domesticus* at room temperature

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(Received 17 February, 2020; Accepted 14 June, 2020)

ABSTRACT

This study aimed to determine the effect of antioxidants onion extract in Ringer's Dextrose diluent on the quality of *Gallus domesticus* sperm during storage at room temperature. Onion extract was made using the maceration method with 50% ethanol as a solvent and evaporated with a *vacuum rotary evaporator* with a temperature of 50 ºC. *Gallus domesticus* sperm was collected using *female teaser* method. The collected semen was divided into 4 treatments, namely T0 (Ringer's Dextrose without onion extract), T1 (Ringer's Dextrose + 0.02 mL of onion extract), T2 (Ringer's Dextrose + 0.04 mL of onion extract) and T3 (Ringer's Dextrose + 0.06 mL of onion extract). Evaluation of semen quality during the storage at room temperature including individual motility, viability, and abnormality were observed within 1, 2, 3, and 4 hours. The experimental design used a Completely Randomized Design (CRD), the data analysis used Variance Analysis (ANOVA) and followed by Duncan's Multiple Range Tests (DMRT). The analysis showed significant differences ($P < 0.01$) for individual motility and ($P < 0.05$) for sperm viability. The best results were shown in T2 (Ringer's Dextrose + 0.04 mL of onion extract). The sperm quality resulted by T2 treatment could last up to 4 hours at room temperature, with an average of individual motility of 50.83 ± 3.76^{b}, viability of 76.28 ± 2.66^{b} and abnormality of 16.87 ± 1.24^{a}. The addition of 0.04 mL onion extract in Ringer's Dextrose diluent within 4 hours at room temperature met the quality requirements to be used in Artificial Insemination (AI).

Key words*: Sperm, Gallus domesticus, Allium cepa, Teaser female, Ringer's Dextrose, Room temperature*

Introduction

Artificial Insemination (AI) is a biotechnology method used to increase chicken productivity. Some storage factors such as temperature, storage time, diluent, and energy sources for spermatozoa are important parameters for the success of AI. Chicken semen that had been collected from males would deteriorate if it was not immediately inseminated. Lubis (2011) reported that chicken sperm could live at room temperature for 30-45 minutes. Therefore, it was needed a semen diluent to maintain the quality of semen.

The problem that appeared in the long process of semen storing was the destruction of the sperm plasma membrane due to the formation of lipid

peroxidation. The contact between semen and the excessive oxygen caused a peroxidative damage. The damage happened due to the fact that the sperm membrane contained many unsaturated fatty acids that were very susceptible to the damage caused by free radical peroxidation. The efforts to maintain the quality and viability of sperm from the damage caused by free radicals could be minimized by the addition of antioxidant compounds. Antioxidant is compound which could convert the existing free radicals into molecules that have less negative impact. Antioxidants reaction with free radicals could minimize the damage of the sperm cell membrane (Iswati *et al.,* 2018).

A natural antioxidant that has not been widely used for the semen dilution is quercetin. Quercetin is a compound that could counteract free radicals. Brüll *et al*., (2015) reported an analysis (% g / 100g) of flavonoids on the onion extract which had, in average, quercetin of 44.2, quercetin dihexoxide of 0.04523, quercetin hexoxide(1) of 0.1557, quercetin hexoxide(2) of 1.79, methylquercetin hexoxide of 0.0481, kaempferol of 0.122, and methylquercetin of 0.0740.

Materials and Methods

Location

The making of onion extract was carried out at the Central Laboratory of Biological Sciences (LSIH) Universitas Brawijaya, Malang. The *Gallus domesticus* semen storage was carried out at the Poultry Installation of the Malang Agricultural Development Polytechnic (POLBANGTAN). For the semen test, it was conducted at the POLBANGTAN Animal and Reproductive Health Laboratory.

Material Research

The making of onion (*Allium cepa*) extract
Onion (*Allium cepa*) was extracted at the Central Laboratory of Life Sciences, Universitas Brawijaya with maceration technique. Onions were peeled and washed using deionized water, then mashed using a blender to form onion juice. One hundred and fifty grams of onion juice was then dissolved in 1 liter of 50% ethanol and stirred for 15 hours using a magnetic stirrer at room temperature. Subsequently, it was filtered using mesh paper number 40. The filtrate obtained was evaporated using a *vacuum rotary evaporator* with a temperature of ± 40ºC. Finally, it was then centrifuged with the rate of 5000 rpm for 10 minutes.

Fresh semen storage of *Gallus domesticus*

The semen from four healthy male *Gallus domesticus*, aged 12 months and weighed of 2.5 kg, was obtained from the Poultry Installation, Malang Agricultural Development Polytechnic. The collection of fresh *Gallus domesticus* semen was carried out using a *female teaser* method. The tools and materials used for semen storage were cotton, 0.9% NaCl, syringe, Eppendorf, and cup which had been equipped with rope for tying it up to a part of the chicken cloaca. The semen storage was carried out every day at 8 o'clock for one chicken.

Preparation of dilution of semen

The *Gallus domesticus* semen was diluted in Ringer's Dextrose (Reg. No: GKL9230500249B1) with different concentrations of onion extract. The tools used for semen dilution were test tubes, test tube racks, 1 ml syringes, micropipettes, and aluminum foil.

Fresh Semen Test and Dilution

Determining the fresh ejaculated semen volume could be done directly by looking at the storage container scale in ml unit. The color of the fresh ejaculated semen could be observed directly with the naked eye. The smell of the fresh ejaculated semen could be observed directly by smelling the smell of the fresh semen and it was known that it had a distinctive chicken odor. Observation of the fresh ejaculated semen pH was carried out using litmus paper. Observation of consistency was carried out directly on the test tube. Mass motility was observed using a microscope with a magnification of 100x. It could be marked by spermatozoa movement waves and would be given criteria of very good (+3) or good (+2) for its mass motility. Individual motility of spermatozoa could be observed using a microscope with 400x magnification and a glass cover. Assessment of individual motility could be seen by how many spermatozoa that moved progressively forward compared to spermatozoa that stayed stationary. The percentage of viability calculation was done by using a smeared preparation with eosin-nigrosine.

$$\text{Viability calculation formula} = \frac{\text{Number of live spermatozoa}}{\text{Number of live spermatozoa + dead spermatozoa}} \times 100\% \quad \ldots (1)$$

Preparations that had been used for viability calculation 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.

$$\text{Abnormality calculation formula} = \frac{\text{Number of abnormal spermatozoa}}{\text{Number of normal spermatozoa + abnormal spermatozoa}} \times 100\% \quad .. (2)$$

The number of spermatozoa per ml of semen was calculated using a *haemocytometer*. The concentration test was done by sucking the semen using an erythrocyte pipette up to 0.05, then adding hypertonic (NaCl) solution up to number 1. The solution was mixed until becoming homogeneous by an eight-forming shaking for 3 minutes. Two to three drops of mixed semen were discarded because they only contained hypertonic fluid. After that, the solution was homogenized again for one minute and another drop was removed. Subsequently, the solution was dropped on a *haemocytometer* and covered with a glass cover. The concentration calculation was carried out under a microscope with a magnification of 400x in five calculation rooms and compiled in the unit of million/mL.

The collected semen was divided into 4 treatments namely T0 (Ringer's Dextrose without onion extract), T1 (Ringer's Dextrose + 0.02 mL of onion extract), T2 (Ringer's Dextrose + 0.04 mL of onion extract) and T3 (Ringer's Dextrose + 0.06 mL onion extract). The evaluation of semen quality during storage at room temperature including individual motility, viability and abnormality were observed within 1, 2, 3, and 4 hours. The experimental design was done by using Completely Randomized Design (CRD), the data analysis used Variance Analysis (ANOVA) and was followed by Duncan's Multiple Range Tests (DMRT).

Result and Discussion

Fresh semen *Gallus dometicus*

The semen volume result in this study was still in the normal range, in which the standard semen volume of chicken ranged from 0.2-0.5 mL (Iswati *et al*., 2018). The semen volume obtained by the teaser female method in this study was greater at 0.5 ± 0.06 (mL) than Herreros (2016) research using massage method which obtained 3.99 ± 100.61[a] (µL). Having pH value of 7.3, this result was in accordance with Iswati et al., (2018) who stated that the pH value of chicken semen was between 7.2-7.6. Mass motility obtained was 3+, as Rochmi and Sofyan (2019) reported that the result of mass motility of fresh semen was +++ (3) ± 0.00. The individual motility and viability resulted were greater at 85% and 94.17 ± 1.47, respectively, compared to Herreros (2016) who obtained 64.67 ± 7.94[a] (%) and 81.00 ± 7.59[a]. The average semen concentration was 354.50 ± 33.72 (10^6 ml) and the abnormality was 5.16 ± 0.75.

Motility of Spermatozoa

Table 1 showed that the control treatment (T0), which was Ringer's Detrose without onion extract addition, could maintain the motility up to 4 hours with an average motility of 40.83 ± 2.04[a]. This result was not significantly different from (T1) and (T3), but it differed significantly in (T2). The T2 treatment was able to maintain the motility for up to 4 hours with the highest motility percentage, i.e. 50.83 ± 3.76[b], amongst other treatments. Ghalehkandi *et al*., (2015) reported that the addition of 15 cc onion extract to the male rats' semen showed a decrease in motility, which was associated with low membrane stability due to the high concentration of onion extract. Ige and Akhigbe (2012) reported that adding 1 ml of onion extract into the male rats' semen obtained the highest motility percentage of 91.8 ± 1.8550[b] and a motility percentage of 45.8 ± 1.8550[d] was obtained in the addition of semen which was exposed to aluminum (AI). Chae *et al*., (2017) stated that quercetin contained in onion (*Allium cepa*) was able to protect against oxidative damage and had a positive effect on the functional parameters of spermatozoa, including viability, and motility.

The best result based on the graph at Figure 1 was found in (T2), namely the addition of 0.04 mL onion extract in Ringer's Dextrose diluent. This showed that there was an effect on sperm motility

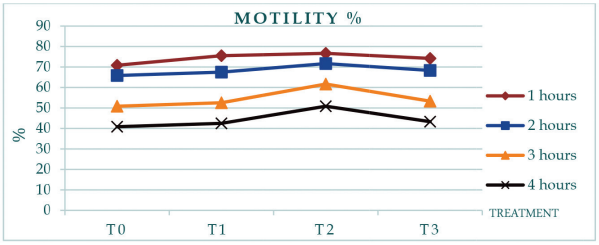

Fig. 1. The graph of individual motility of *Gallus domesticus* sperm with different concentrations of onion extract diluted in Ringer's Dextrose within 4 hours at room temperature (%).

with the addition of 0.04 ml onion extract within 4 hours storage at room temperature. Chae et al., (2017) stated that the effect of adding onion extract (50 µg/mL) on human sperm which was observed after 3 hours of incubation showed 66.0% greater sperm motility than the control group which showed 54.0% sperm motility.

Viability of Spermatozoa

ANOVA test showed at Table 2 there was a significant effect in the viability of spermatozoa ($P < 0.05$) due to the treatment of adding different concentrations of onion extract in Ringer's Dextrose diluent, within 4 hours of storage at room temperature. Within 4 hours of storage, (T2) treatment showed the highest average viability results compared to T0, T1 and T3, i.e. 89.99 ± 3.93^b, 88.99 ± 3.36^b, 86.99 ± 2.32^b, and 76.28 ± 2.66^b. The addition of 0.04 mL onion extract in Ringer's Dextrose diluent was more effective in maintaining viability compared to T0, T1 and T3 treatments. This indicated that onion extract could reduce free radicals caused by oxidative stress during room temperature storage, which could prevent the sperm damage. Khaki *et al.*, (2015) reported that the addition of 1g onion extract in rats' semen obtained greater viability at 96.20 ± 1.20*, while 0.5g addition obtained 93.60 ± 1.83* viability, compared to control treatments which obtained 66.25 ± 4.73. Chae *et al.*, (2017) stated that quercetin contained in onion (*Allium cepa*) was able to protect against the oxidative damage and had a positive effect on the functional parameters of spermatozoa, including viability.

Figure 2 showed the sperm cell death indicator was if the sperm cells absorbed the eosin-nigrosine staining. Meanwhile, the live sperm cells would not absorb the staining. Kondracki (2017) stated that live sperm cells would not absorb the staining because the plasma membrane would be undamaged so that it could protect cells. On the other hand, the dead spermatozoa would absorb the staining since their plasma membrane were damaged.

Abnormality of Spermatozoa

ANOVA test showed at Table 3 there was significant difference ($P < 0.05$) to the percentage of spermatozoa abnormality as the result of the treatment of adding the different concentrations of onion extract in Ringer's Dextrose diluent, within 4 hours of storage at room temperature. During 4 hours of storage, (T2) treatment resulted the lowest average

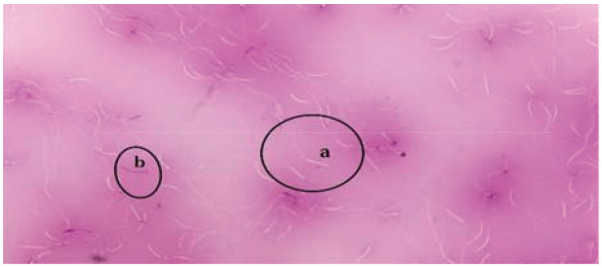

Fig. 2. Viability spermatozoa *Gallus domesticus* by eosin-nigrosine staining (a) live sperm; (b) dead sperm

Table 1. The average of sperm motility of *Gallus domesticus* in the different concentration of onion extract diluted in Ringer's Dextrose at room temperature (%)

Group of treatments (mL)	Observation time (h)			
	1	2	3	4
T0 (0)	70.83 ± 2.04^a	65.83 ± 2.04^a	50.83 ± 2.04^a	40.83 ± 2.04^a
T1 (0.02)	75.50 ± 2.74^a	67.50 ± 2.74^a	52.52 ± 2.74^a	42.50 ± 2.74^a
T2 (0.04)	76.67 ± 2.58^b	71.67 ± 2.58^b	61.67 ± 2.58^b	50.83 ± 3.76^b
T3 (0.06)	74.17 ± 3.76^a	68.33 ± 2.58^a	53.33 ± 2.58^a	43.33 ± 2.58^a

Table 2. The average viability of sperm *Gallus domesticus* with different concentration of onion extract diluted in Ringer's Dextrose at room temperature storage (%).

Group of treatments (mL)	Observation time (h)			
	1	2	3	4
T0 (0)	86.03 ± 3.08^a	84.06 ± 2.60^a	81.98 ± 3.71^a	73.90 ± 2.76^a
T1 (0.02)	86.06 ± 2.93^a	85.42 ± 2.75^a	83.72 ± 2.71^a	73.13 ± 1.78^a
T2 (0.04)	89.99 ± 3.93^b	88.99 ± 3.36^b	86.99 ± 2.32^b	76.28 ± 2.66^b
T3 (0.06)	83.96 ± 1.26^a	83.72 ± 1.17^a	82.76 ± 1.22^a	72.30 ± 1.92^a

Table 3. The average abnormality of *Gallus domesticus* sperm with different concentrations of onion extract in Ringer's Dextrose diluent at room temperature storage (%).

Group of treatments (mL)	Observation time (h)			
	1	2	3	4
T0 (0)	7.71±0.58[c]	8.34±0.50[a]	10.72±0.92[a]	16.89±1.01[a]
T1 (0.02)	6.87±0.53[b]	8.00±0.43[a]	10.57±0.90[a]	17.50±1.38[a]
T2 (0.04)	6.11±0.34[a]	7.35±0.60[a]	10.53±0.79[a]	16.87±1.24[a]
T3 (0.06)	7.40±0.84[b]	8.72±1.31[b]	10.25±1.26[a]	17.83±1.05[a]

abnormality compared to T0, T1 and T3, i.e. 6.11 ± 0.34[a], 7.35 ± 0.60[a], 10.53 ± 0.79[a], 16.87 ± 1.24[a]. Khaki *et al*., (2015) reported that the addition of 1g onion extract to the rats semen obtained fewer abnormality at 5.12 ± 0.656 and 0.5g of onion addition obtained 5.20 ± 0.618 abnormality, compared to the control treatment which obtained 6.27 ± 0.711. Iswati *et al*., (2018) stated that high spermatozoa abnormality would affect the fertilization. Hence, low abnormality is an AI requirement which is also influenced by some factors, those are abnormalities are age, temperature, maintenance management, dilution and environment.

Figure 3. showed the abnormality which was indicated by the broken tail and the bent base tail. Danang *et al*., (2012) stated that the abnormality of the head occurred during spermatogenensis in seminiferous tubules and the abnormality of the tail occurred due to dilution and environmental-related factors, which is included the storage.

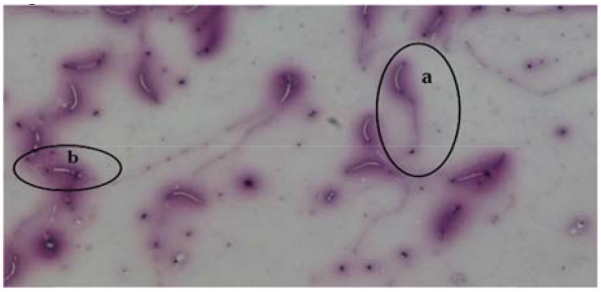

Fig. 3. Sperm abnormality of *Gallus domesticus* (a) normal sperm; (b) abnormal sperm

Conclusion

In conclusion, the making of onion extract by maceration method could be done by using 50% ethanol solvent and a vacuum rotary evaporator. The analysis showed a significant difference (P <0.01) for individual motility and (P <0.05) for sperm viability. The best results were shown in T2 treatment (Ringer's Dextrose + 0.04 mL of onion extract). The sperm quality resulted by T2 treatment could last up to 4 hours at room temperature, with an average of individual motility of 50.83 ± 3.76[b], viability of 76.28 ± 2.66[b], and abnormality of 16.87 ± 1.24[a]. Therefore, it could be concluded that the addition of 0.04 ml onion extract in Ringer's Dextrose diluent within 4 hours at room temperature met the quality requirements for use in Artificial Insemination (AI).

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