Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 20, No. (1) : 2018 : 12-20

ISSN-0972-3005

EFFECT OF ADDITION OF GLUTATHIONE IN DILUENT RINGER'S ON SPERMATOZOA QUALITY OF DOMESTIC CHICKEN DURING COLD STORAGE

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(Received 1 May, 2017; accepted 15 July, 2017)

Key words : *Teaser female, Sperm, Domestic chicken, Glutathione, Ringer's*

Abstract - Domestic chicken is a local chicken of Indonesia which is the local genetic resource and should be preserved because it has the advantage of high adaptability to the environment. This study aimed to determine the effect of the antioxidant glutathione in Ringer's diluent to on sperm quality of local chicken during storage at 3-5°C. Maintenance and semen collection of domestic poultry with method teaser female performed at the College of Agricultural Extension (STPP) Malang. Semen of chicken result shelter was divided into 4 treatment that P0 (Ringer's without glutathione), P_1 (Ringer's+0.1 mM glutathione), P_2 (Ringer's + 0.5 mM glutathione) and P_3 (Ringer's + 1.0 mM glutathione) with a concentration 200x106 sperm cells/mL each treatment. Evaluation of semen quality during cold storage include individual motility, viability, and sperm abnormality was observed at hour 0, 4, 8, 24, 32 and 48. The experimental design used randomized block design group nested patterns, analyzes the data using Analysis of Variance (ANOVA). The analysis showed that addition of glutathione in the Ringer's diluent showed significant differences ($P < 0.05$) on individual motility, viability and sperm abnormality in domestic poultry. The best results are shown in the treatment of P_2 (Ringer's +0.5 mM glutathione). Sperm quality can be preserved in cold storage until 8 hours of P_2 treatment, with a mean of individual motility 62 ± 2.91%, viability 79.26 ± 2.68% and 13.66 ± 0.31% abnormality. The addition of 0.5 mM glutathione in Ringer's diluent and storage up to 8 hours at a temperature of 3-5 °C, they meet quality requirements for use in artificial insemination. It is recommended that further research to determine fertility and hatchability of eggs of domestic poultry were inseminated using semen diluted using Ringer's + 0.5 mM glutathione with old cold storage up to 8 hours.

INTRODUCTION

Artificial Insemination (AI) success factor among others influenced by the quality of semen, environmental maintenance, engineering and precise timing to AI, AI dose and skills inseminator. Semen quality is determined by several factors including: genetic males, physiological (libido and age males), methods of semen collection, semen hygiene, frequency of collection semen and diluents and cement storage (Irastuti 2011; Wiyanti, *et al*., 2013).

Semen chickens that have been collected from selected sires will soon deteriorate if not immediately inseminated in hens, as presented by Lubis (2011) that sperm chicken can live at room temperature for 30-45 minutes, so we need storage by additional diluents of semen to maintain the quality of semen so as to achieve high fertility. Storage can be done is a cold storage at a temperature of 3-5 °C which aims to inhibit the metabolic activity of sperm both physically and chemically. The quality of sperm during storage should be evaluated to determine the extent of the power of life and fertility of spermatozoa in the female reproductive tract. By knowing the best storage time, the semen quality can be maintained and the use of male more efficient (Danang *et al*., 2012).

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The success of the AI in chickens depends on the diluent used. Danang, *et al*. (2012) reported that semen storage chickens at 4 °C by using Ringer's were able to maintain the quality of semen maximum at 18 hours, because solution of Ringer's containing various mineral salts that have a buffer and isotonic to support the motility of sperm in a longer time. Similar delivered by Ridwan and Rusdin (2008) that the Na-lactate Ringer's necessary to meet the needs of bicarbonate ions that serve to maintain the acidity of the solution or as a buffer solution.

The problems that arise in the process of cold storage semen is sperm plasma membrane damage due to the formation of lipid peroxidative. Contact between semen and excessive oxygen cause damage peroxidative. The damage caused by the sperm membrane contains a lot of unsaturated fatty acids which are very susceptible to damage caused by free radical peroxidation. Efforts to maintain the quality and viability of sperm from damage caused by free radicals can be minimized by the addition of antioxidant compounds. Antioxidants are compounds that can alter the existing free radicals into molecules that have a less negative impact, one antioxidant is glutathione. Antioxidants react with free radicals so as to minimize damage to the sperm cell membrane (Surai, *et al*., 2001; Rizal and Herdis, 2008).

Glutathione (GSH) is an antioxidant sulfhydryl (-SH), antitoxin and enzyme cofactor that has the properties neutralize free radicals. Glutathione is one of the antioxidants that play a role in the protection section of the sperm cell plasma membrane damage due to the toxic nature caused by reactive oxygen species (Reactive Oxygen Species, ROS) (Rizal and Herdis, 2010). The use of glutathione has been much research done on semen mammals, although not yet widely applied in semen chicken. Research by Thananurak, *et al*., (2015) by adding glutathione on semen freezing chicken with a concentration of 0.1 mM, 0.5 mM and 1 mM and post-thawing semen examination has not shown an increase in semen quality. However, so far unknown addition of glutathione during cold storage of semen chicken. The addition of the antioxidant glutathione in chicken semen dilution is expected to keep semen quality of chicken during cold storage.

Under these conditions, it is necessary to do research on the effect of glutathione in Ringer's diluent basis of the quality of spermatozoa during the cold store, in order to obtain the proper concentration of glutathione which can extend the shelf life of chicken cement in temperatures of 3-5 °C.

MATERIALS AND METHODS

Location

The study was conducted at the College of Agricultural Extension (STPP) Malang. Maintenance domestic chicken and collection semen in the Installation Poultry STPP Malang while evaluation fresh semen and chilled semen of conducted at the Laboratory of Reproductive and Animal Health STPP Malang.

Material Research

This research used the native chicken(Gallus domesticus) sex male, 1-year-old, had a body weight of 2.5 kg. The experimental animals should be a healthy reproduction and have a high libido as evidenced by the desire to climb the females if brought closer to the hen. The collection of semen from two roosters and one hen as a teaser, caged individually. Feed is given in the form of commercial feed (pellets) and the drinking water supplied adlibitum. Semen collection is done 2 times a week using a teaser female method.The cement used in the study has a value of mass motility minimum + 2 and percentage motility = 70%.

Semen collection is done in a way feathers around the cloaca be shaven, the area around the cloaca cleaned of dirt with a tissue soaked with disinfectant. Males was fitted a tube cloaca artificial as an artificial vagina and then brought closer to the female and male anglers will happen response to climb females, a few seconds the ejaculation of semen occurs and the semen is collected in tube artificial cloaca. Strap tube removed and the semen is aspirated with a syringe 1 cc to know the volume of semen, then poured into Eppendorf's tubes for examination of fresh semen.

Semen diluent

Materials diluent used is Ringer's solution (Ringer lactate) having the composition per 1000 mL are: Sodium chloride 6.0 g, Sodium Lactate 3.1 g, Potassium Chloride and Calcium Chloride 0.3 g 2H2O 0.2 g (Danang *et al*., 2012) and glutathione antioxidant ingredients (Jincheng Pharm; CAS 70-18-8). Dilution treatment is Ringer's without glutathione (P0), Ringer's + 0.1 mM glutathione (P1),

Ringer's + 0.5 mM glutathione (P2), Ringer's + 1 mM glutathione (P3). Fresh cement is divided into four test tubes with a concentration of 200 million/mL. The test tube which already contains semen and diluent with various doses of glutathione put in a glass beaker filled with water that is clean as a water jacket, and then stored in a refrigerator temperature of 3-5 °C.

Examination of the quality of semen

The quality of fresh semen is observed immediately after the collection includes examination of macroscopic: volume (mL), colour, consistency, pH and microscopic examination included mass motility, the motility of the individual (%), viability (%), concentration (x10^6/ mL) and abnormality (%) (Susilawati, 2011; Ismaya, 2014). Measurement Volume of semen is done by looking at the scale of 1 cc syringe. Colour of semen is viewed directly on the tube container. Observation of consistency by tilting the tube slowly and views semen viscosity. Measurement of pH using a pH meter paper and color is matched with pH indicator.

Mass motility is checked immediately after the semen is collected with dripping fresh semen above the glass object is then observed with a magnification of 100x. Individual motility examination is done by putting semen on top of the object glass and closed the cover glass and observed under a microscope magnification of 400x. Calculation of concentration was done by using hemocytometer counting chamber. Viability examination was performed by eosin staining-nigrosin (Susilawati 2011; Ax *et al*., 2008).

Evaluation of semen after dilution is done on the clock to 0,4,8,24,32 and 48. The observed variables include the percentage of individual motility, viability and sperm abnormality. Rating individual motility percentage (%) with a direct view of preparations at glass object 400x magnification with a scale of 0-100% votes. The percentage of viability (%) obtained by counting the number of spermatozoa are alive or not absorb the color of eosin-nigrosin divided by total spermatozoa are counted x 100%. Obtained by calculating the percentage of spermatozoa abnormalities abnormal divided by the total spermatozoa were examined x 100%.

The data analysis

The research design used was a randomized block design nested pattern with 4 treatments dilution in six long shelves (hours 0,4,8,24,32, and 48) at temperature 3-5°C and each treatment 10 replicates. Data were analyzed used by Analisys of Variance (ANOVA) and Microsoft Excel and continued by Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Semen of domestic chicken of the collection by using teaser female in the study then examined macroscopically covering volume, color, pH, and consistency, as well as microscopic examination covering the mass motility, individual motility, concentration, viability, and abnormality. Results of Semen quality examination of this research can be seen in Table 1.

Table 1. Quality of domestic Chicken Semen

Parameters	Standard	Mean ± SE
Macroscopic		
Volume (mL)	0.2-0.5	0.56 ± 0.029
Color	White milk	White milk
pH	7.2 to 7.6	7.50 ± 0.167
Consistency	-	viscous
Microscopic		
mass Motility	+2	+3
Individual Motility (%)	60-80	83.00 ± 1.527
concentration (million/mL)	3000-7000	4175 ± 232, 750
Viability	(%)>70%	89.12 ± 1,577
Abnormality (%)	10-15	10.27 ± 0.527

Table 1 indicates that the quality of the semen obtained from 10 replicates is in a normal condition. This is in accordance with the opinion of Garner and Hafez (2008) which is used as a standard for comparison of data in the Table above. The volume of cement in the research is still in the normal range ranged between 0,2 0,5 mL (Garner and Hafez, 2008). The color of semen in the study is milky white, it is considered normal as reported by Peters *et al*.,(2008) that the color of chicken semen is milky white. In collection semen with the female teaser method is not found in the semen abnormal discoloration of chicken, this was due to lack of manipulation of the hands of collectors at the time of the shelter stimulation occurring naturally.

Examination of pH with pH paper shows the results of 7.5, this results in accordance with Garner and Hafez (2008) that the chicken semen pH between 7.2-7.6. This is in line with the statement of Peters *et al*., (2008) that the chicken semen was

slightly alkaline and reports Donoghue and Wishart (2000) that the pH of chicken semen ranging between 6.0- 8.0. In this study, the semen used has an average viscous consistency. The consistency of semen has a correlation with sperm concentration, the more the number of spermatozoa in the semen consistency is more viscous (Ax *et al*., 2008; Susilawati, 2011).

Mass motility +3 are very well seen by examination use a microscope resembles a thick wave of cloud, while +2 is good appraisal which saw a wave that is thinner and moves slowly. As this research standard cement with a mass of at least + 2 decent motility further processing (Susilawati, 2011). The average of the individual motility of fresh semen in this study was 83.0 ± 4.830 (%), these results meet the criteria of fresh semen that can be processed for a minimum of 70% dilution is progressively moving sperma- tozoa.

The percentage of spermatozoa Viability of fresh semen in this study was 89.12 ± 4.988 (%), it included a good assessment as presented by Lukman *et al.*, (2014) that the viability of spermatozoa between 60-75%. Evaluation of fresh semen abnormalities was used in this study was 10.27 ± 0.53 (%), it is said to be good because of below 20%, according to Susilawati (2011) and Ismaya (2014) if both primary and secondary abnormalities over 20 % have a lower conception. Meanwhile, according to Garner and Hafez (2008) cement abnormal morphology good chicken ranged 10-15%.

The percentage of sperm motility

Percentage of motile spermatozoa is the most important variable in determining the quality of semen (Rizal and Herdis, 2008). The quality of the semen sample and the indicator of the ability spermatozoa can be shown through assessment of sperm motility (Getachew, 2016). Motility is one important measure that demonstrates the ability of sperm to fertilize the ovum in the process of fertilization (Danang et al.,2012; Zahariev, 2007). A good quality of spermatozoa shown by the high progressive motility that will determine the speed up on the site of fertilization. Individual motility of spermatozoa of domestic poultry in several concentrations of glutathione during cold storage temperature of 3-5 °C decreased gradually. Mean motility with different concentrations of glutathione during storage at 3-5 °C are listed in Table 2.

Results of ANOVA showed that different diluent significant (P <0,05) on individual motility of spermatozoa of domestic poultry during the cold storage. Duncan test showed that on a long shelf-hour-0, control treatment P_0 is significantly different to the treatment of P_2 and not significantly different from P_1 and P_3. In the old storage hours 4th, all treatments showed motility are still good in which P_2 at the highest rate and significantly different from P_0, P_1, and P_3. In the old store hours 8th, treatment P_3 have started to show a decrease in individual motility quality for less than 40% and significantly different from P0, P_1 and P_2.

At 24 hour storage and 32 hour, only P_2 are still able to maintain a good percentage of individual motility. In the old store 48 hours, all treatments showed individual motility below 40%, which at P0 was not significantly different with P1 and P3 but significantly different from P_2.Overall, on a long shelf-hour-0 until the 48th hour, the treatment of P_2 shows the percentage of the highest individual motility compared to other diluents. Treatment P_2 may significantly better results on sperm motility domestic poultry.

Motility was kept longer showed a decrease in motility, it is they are due to the adaptation process of spermatozoa with diluent and cooling processes that take place can affect metabolic activity. (Lukman *et al*., 2014). The cooling process causes a decrease in motility of sperm cells because of a shock (cold shock). Sperm cells are exposed to cold shock would sooner die, because of the availability of energy in the diluent diminishing returns and

Table 2. Mean sperm motility Native Chicken with Glutathione Different concentrations in Ringer's diluent During the Cold Storage (%)

Dilution	0	4	8	24	32	48
P_0	71±2.77^{ab}	53±2.60^{b}	42±3.59^{b}	25±3.42^{b}	22±3.26^{b}	16±2.67^{ab}
P_1	74± 2.67^{ab}	56±4.99^{b}	46±5.42^{b}	37±3.96^{c}	29±3.48^{b}	16±2.67^{ab}
P_2	77± 1.53^{b}	67±3.00^{c}	62±2.91^{c}	45±4,28^{c}	41±5.26^{c}	25±2.69^{c}
P_3	67± 3.5^{a}	48±3.27^{a}	29±3.78^{a}	13±1.15^{a}	9±1.00^{a}	8±1.33^{a}

Description: different superscript in the same column indicate significant differences (P <0,05)

increasing the acidity of the semen due to the dead spermatozoa produce lactic acid and dead sperm will lose the ability motility (Solihati, 2006). The decline in the percentage of sperm motility during storage because of the energy used by sperm derived from glyceryl-phosphorylcholine, fructose and sorbitol contained in semen (Garner and Hafez, 2008) will gradually be used for metabolism and slowly going downhill. Besides this, also occur during cold storage and physical and chemical stress on the membrane of spermatozoa which can reduce the ability of sperm fertilization. Oxidative stress also occurs during the cold store which caused a decrease in motility and increased the percentage of dead sperm cells (Khan, 2011).

Control treatment (P0) Ringer's without glutathione can maintain motility until the 32 hours with an average motility of 42 ± 3.59%, the rate is not significantly different from P_1, but significantly different from P_2 and P_3. The dilution of Ringer's treatment is different from the results reported by Danang *et al*.,(2012) that the motility of chicken can be maintained until all 18 are 47.0 ± 5.87% at storage temperature of 4 ° C.

Treatment P_1 can maintain motility up to 8 hours with an average of 46 ± 5.42%, these results were not significantly different at P_0, but significantly different from P_2 and P_3. Treatment P_2 can keep up to an hour to-motility 32 with an average of 41 ± 5.26%, this figure is evident with the difference treatment P_0, P_1 and P_3. Ability to maintain of motility the longest during a cold storage of P_2 shows the concentration of glutathione is the best. This is in contrast with the Treatment P_3 to maintain the motility only till to-4 with an average of 48 ± 3:27%, which different real with P_0, P_1 and P_2, and the ability to maintain sperm motility minimum for cold storage. It is also reported by Thananurak (2015) that the addition of 1mM Glutathion to chicken cement thawing media showed a decrease in motility, which is associated with low membrane stability due to the high concentration of glutathione (Buty *et al*., 2002) although it can inhibit ROS formation during storage.

The longer the storage energy for the movement of spermatozoa from semen is exhausted, thus requiring reserve energy from the mitochondria. Garner and Hafez (2008) revealed that in order to maintain motility, spermatozoa require energy derived from the overhaul of Adenine triphosphate (ATP) in the mitochondrial sheath that results in Adenine diphosphate (ADP) and Adenine monophosphate (AMP), energy produced under normal circumstances as energy Motion and when not in use will be lost as heat. Mitochondrial integrity can help in maintaining motility. The exact dose of P_2 is assumed to prevent mitochondrial damage from ROS effects, so that it can maintain longer motility.

The addition of the enzymatic antioxidant glutathione plays a key role in the detoxification of lipid peroxide each appearing in chicken spermatozoa and was instrumental in the conversion of hydrogen peroxide into less harmful components (Khan, 2011). Low doses of glutathione at P1treatment (0,1mM) are not able to stabilize free radicals in the semen stored at 3-5 °C. Because there is no balance between antioxidants with ROS, ROS increasing and low antioxidant cause oxidative stress resulting in increased lipid peroxidation and decreased motility (Khan, 2011). This is supported by the opinion of Wang *et al*., (2003) found a high ROS production will damage the mitochondria of sperm cells, where the mitochondria have a role in cell motility, resulting in the absence of antioxidant balanced against ROS cause fertilization ability decreases.

Conversely, excessive doses of glutathione causeat P_3 tratment (1mM) a decrease in the percentage of progressive motility of spermatozoa. Addition of glutathione in excessive cement thinners assumed would lead to saturation of antioxidants as submitted by Lukman *et al*.,(2014) administration of a-tocopherol excessive dilution causes saturation of antioxidants in Bali cattle semen stored at 5 °C. The same thing also reported by Tananurak (2015) that administration of 1 mM glutathione in dilution decreases the motility post-thawing the frozen semen of chicken. The appropriate dose in chicken semen dilution for optimum counteracting free radicals during storage temperature of 3-5 ° C is 0.5 mm (P_2), which is expected to increase the fertility of sperm chicken. Similar feelings were expressed by Ridwan and Rusdin (2010) that the concentration of glutathione in the proper semen thinners will improve or maintain the quality of sperm morphology and motility.

The percentage of sperm viability

Semen quality factors that determine the success of artificial insemination, among others viability of spermatozoa. Viability is important because only

spermatozoa survive in the female reproductive tract that is able to reach the site of fertilization and fertilize the ovum. The addition of glutathione in Ringer lactate diluent during the cold store is able to maintain the viability of spermatozoa, yet the percentage of viability declines as a long shelf cement during cooling. The mean percentage of spermatozoa viability of domestic poultry from each treatment during the storage temperature of 3-5 °C are presented in Table 3.

Results of ANOVA showed that the treatment of different diluents during 3-5 °C storage temperature significantly affect the viability of spermatozoa ($P < 0.05$). Duncan test showed that at the 0th all treatments showed no significant differences, with treatment P_1 on the percentage of the highest viability. In the old store hours 4th until the 48th hour, Treatment P_2 shows the highest number compared to P_0, P_1, and P_3. The addition of a diluent glutathione 0.5 mM in Ringer's solution is more effective in maintaining the viability compared with other treatments. This suggests that glutathione can reduce free radicals caused by oxidative stress during temperature storage 3- 5 °C, thus preventing damage to sperm that can reduce the percentage of spermatozoa chicken. Similar feelings were expressed by Triwulaningsih (2003) that administration of 0.5 mM glutathione in cattle semen dilution medium gives the best results on the viability during storage 5 °C. Treatment of lower glutathione concentration or excessive thinning Lactate Ringer will decrease the percentage of sperm viability.

Ax *et al.*,(2008) and Lukman *et al.*,(2014) states that the percentage of sperm viability minimum standards that can be used for artificial insemination is around 60-75%. In the treatment of P0 viability of spermatozoa can be maintained until the 8th ie 64.2 ± 2.31% and was significantly different from the P_2 and P_3, but not significantly different from P_1. Treatment P_1 is able to maintain spermatozoa until the 24th hour on the numbers 61.9 ± 1.89%, which was not significantly different at P_2, but significantly different from P_0 and P3.Treatment P_2 is able to maintain viability until the 32nd at 60.6 ± 4.16%. Treatment P_3 only able to maintain viability until the 4th and this is the shortest survival among another diluent. At a concentration of 0.5 mM glutathione longer able to maintain viability because of the presence of glutathione concentration in the diluent right Ringer's capable of inhibiting free radicals during cold storage temperature of 3-5 °C.

Table 3, indicating that the longer the shelf life at 3-5 °C decreases the percentage of sperm viability in various concentrations of glutathione. Loss of viability of spermatozoa during storage occurs due to damage that causes the membrane into the cell osmotic pressure (Hakim, 2014). Damage to sperm cell membrane permeable membranes has an impact on the nature of those who are not able to select the entry and exit of the dye eosin-nigrosin into the plasma. Overview live and dead spermatozoa shown in Figure 3. Cells live spermatozoa do not absorb colors because of the plasma membrane intact as a protective cell, whereas dead spermatozoa had damaged its plasma membrane, thereby absorbing colors (Susilawati, 2011).

The percentage of sperm abnormality

Abnormal morphology of sperm is highly correlated with power cow fertility, abnormalities of cattle more than 20% can reduce the ability of spermatozoa fertilization (Susilawati, 2011). According to Garner and Hafez (2008) on the nation's poultry morphologically normal spermatozoa must meet a percentage of about 85-90%, which means abnormal spermatozoa should not be more than 15% to be used for insemination. Average of spermatozoa abnormalities of each treatment during 3-5 °C storage temperature shown in Table 4.

Table 4 shows the average percentage of abnormalities in the diluent P_0,P_1,P_2 and P_3 for a storage temperature of 3-5 °C. Based on the ANOVA showed significant dilution treatment ($P < 0.05$) on sperm abnormalities range hens during the storage temperature of 3-5 °C. Duncan test

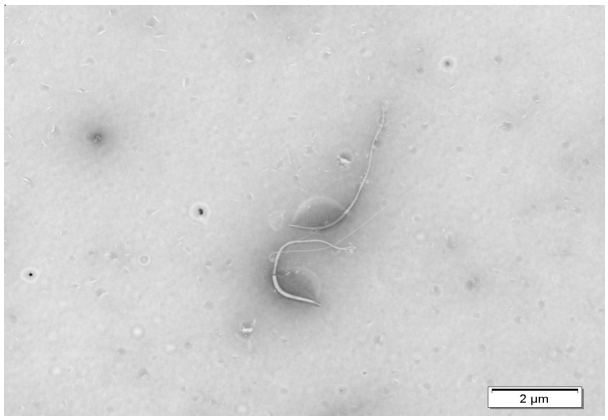

Fig. 1 Viability Spermatotoza domestic chicken by-nigrosin eosin staining (a) live sperm; (b) dead sperm

Table 3. Mean Sperm Viability of Native Chicken with Glutathione Different concentrationsin Ringer's diluent During the Cold Store (%)

Dilution	0	4	8	24	32	48
P_0	82.64±3.21	70.26±2.96^{ab}	64.2±2.31^{b}	49.54±3.76^{b}	43.18±4.16^{b}	32.19±2.46^{ab}
P_1	88.88±1.72	79.09±1.22^{bc}	73.05±1.96^{bc}	61.9±1.89^{c}	46.22±4.04^{b}	34.55±4.11^{ab}
P_2	86.78±2.03	81.93±1.67^{c}	79.26±2.68^{c}	67.85±2.15^{c}	60.6±4.16^{c}	46.13±5.29^{c}
P_3	83.01±1.63	64.02±4.15^{a}	50±5.24^{a}	36.22±5.23^{a}	31.38±5.71^{a}	25.16±4.16^{a}

Description: different superscript in the same column indicate significant differences (P <0,05)

Table 4. Mean sperm abnormalities on Native Chicken with Glutathione concentration in Ringer lactate diluent during cold store (%)

Diluent	0	4	8	24	32	48
P_0	12.55±0.6^{ab}	18.92±1.75^{b}	21.08±1.12^{b}	23.87±0.08^{b}	26.59±0.87^{b}	26.86±0.68^{ab}
P_1	12.46±0.52^{ab}	14.33±1.20^{a}	21.30±1.47^{b}	24.47±0.94^{bc}	27.84±0.81^{b}	31.28±0.88^{c}
P_2	11.3±0.44^{a}	12.91±0.33^{a}	13.66±0.31^{a}	20.85±0.67^{a}	21.16±0.63^{a}	25.13±0.71^{a}
P_3	13.53±0.49^{c}	19.67±1.41^{b}	24.57±1.83^{c}	29.7±1.78^{c}	30.88±1.02^{c}	33.65±0.97^{c}

Description: different superscript in the same column indicate significant differences (P <0,05)

showed that the long storage hours and hours of all 0 to 4, all treatments showed abnormalities that meet the standards. In the old store hours 8th, Treatment P_2 showed abnormalities of the lowest compared to other treatments, and these results are significantly different from P_0, P_1 and P_3. In the old save until the 24th hour to the 48th hour All treatments showed abnormalities averaging above 20% during storage at 3-5 °C, so long shelf were not eligible for artificial insemination.

Abnormalities classified into primary and secondary abnormalities. The primary abnormality occurs during the process of spermatogenesis related to the head and the acrosome, while secondary abnormalities occur after the process of spermatogenesis, when ejaculation, shelter, semen evaluation and processing of cement. Primary abnormality generally in the form of abnormalities in the head (too big or small, the head of more than one), the tail of two(doubletail). Secondary abnormality characterized by the tail broken off, tail coiled and twisted piece mid section (Ax *et al*., 2008; Susilawati, 2011; Rizal and Herdis, 2008).

Treatment P_0, P_1, P_2, and P_3 at the old store 48 hours showed the highest abnormality that during the cold store that is equal to 26.86 ± 0.68%; 31.28 ± 0.88%; 25.13 ± 0.71% and 33.65 ±0.97%. Treatment P3 at the 48th hour is the highest among the abnormalities of diluent and other long shelf life. Abnormalities were found in this study is a round shaped head, the head is larger, severed heads, tails curled. The longer the storage time in cold temperatures, the higher the percentage of abnormal spermatozoa. This was disclosed by Solihati *et al*., (2006), the longer the storage will be growing spermatozoa die because of damage to the plasma membrane of spermatozoa thus increasing abnormal spermatozoa.

Treatment of P_0, P_1 and P_3 shows the percentage of abnormality less than 20% for only 4 hours. While P_2 treatment is able to give percentage of abnormality less than 20% until 8 hours namely by averaging 13.66 ± 0:31%, demonstrating the ability to maintain the percentage of morphologically normal longest at storage temperature of 3-5 °C. Spermatozoa with an abnormally high will affect fertilization so that the low abnormality Traffic is a prerequisite for quality of spermatozoa for insemination. Factors affecting genetic abnormalities in spermatozoa among other things, breed, age, light and temperature, maintenance management, frequency shelter, dilution and the environment.

Visualization of domestic chicken poultry spermatozoa abnormalities with eosin-nigrosin staining is presented in Figure 2.

Figure 2 shows the abnormalities of the domestic chicken spermatozoa seen in the head region, mid-piece or tail, there is rounded head (d), bending head (f), midpiece bending (b, h), midpiece broken off (c), base of tail bending (g), and tail bending (e). Head abnormalities occur during

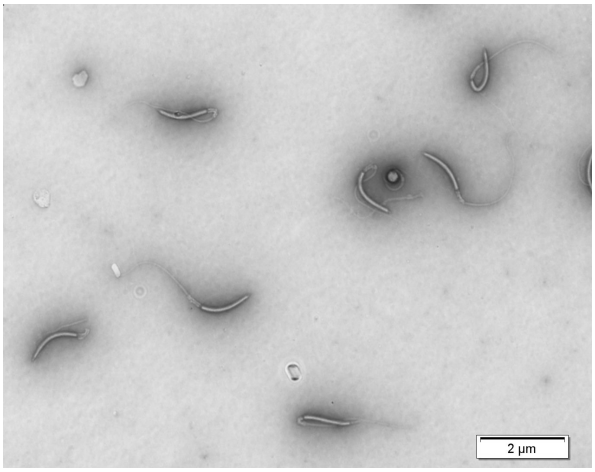

Fig. 2 Sperm abnormality of domestic chicken, normal spermatozoa (a), abnormal spermatozoa (b,c,d,e, f,g,h).

spermatogenesis in tubuliseminiferus whereas tail abnormalities occur due to dilutions and environmental factors including storage and cooling processes (Danang *et al.,* 2012).

CONCLUSION

The addition of glutathione antioxidant in Ringer's diluent is able to maintain the quality of the domestic poultry spermatozoa during storage temperature of 3-5 °C. The addition of 0.5 mM glutathione in Ringer's diluent is an appropriate concentration in maintaining the quality of the domestic poultry spermatozoa until the 8th 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 find out fertility and hens power of chicken egg that is inseminated by semen diluted with Ringer's + 0.5mM glutathione with cold save up to 8 hours.

ACKNOWLEDGEMENT

Acknowledgments to STPP Malang, especially Poultry Installation and Animal Reproduction Laboratory and Animal Health which has been supported by providing location, facilities and research material. The author also conveyed his remarks to the STPP Malang and Development Agency for Agricultural Human Resources, the Ministry of Agriculture, Republic of Indonesia for the support of the scholarship fund.

REFERENCES

Ax, R.L., Dally, M.R., Didion, B.A., Lenz, R.W., Love, C.C., Varner, D.D., Hafez, B. and Bellin, M.E. 2008. *Semen Evaluation in Farm Animal Reproduction* ed by Hafez ESE. 7 th edition. Lea and Febiger. 365-375.

Buty, B.A., Hampton, M.B. and Winterbourn, C.C. 2002. Detection of oxidant sensitive and thiol proteins by fluorescence labeling and two-dimensional electrophoresis. *Proteomics*. 2 : 1261-1266.

Chelmonska, B., Jerysz, A., Ukaszewicz, E.L., Kowalczyk, A. and. Malecki, I. 2008. Semen Collection from Japanese Quail (*Coturnix japonica*) Using a Teaser Female. *Turk. J. Vet. Anim. Sci.* 32 (1) : 19-24

Danang, D.R., Isnaini dan, N. and Trisunuwati, P. 2012. Save Old influence on the Quality of Sperm Semen Native Chicken In Ringer's diluent at 4 °C. *Jurnal Ternak Tropika*. 13 (1) : 47-57 (Indonesia).

Donoghue, A.M. and Wishart, G.J. 2000. Storage of poultry semen. *Animal Reproductive Science.* 62 : 213-232.

Garner, D.L. and Hafez, E.S.E. 2008. *Spermatozoa and Seminal Plasma in Reproduction in Farm Animals*. Edited by E.S.E. Hafez and B. Hafez. 7th edition. Lea and Febiger. Philadelphia: 167-188

Getachew, T. 2016. A Review Article of Artificial Insemination in Poultry. *World's Veterinary Journal*. 6 (1) : 26-35.

Irastuti, 2011. Effect of Nations males and Interval Deposition Semen terhadap Artificial Insemination Success in chickens. *Journal of Science and Technology Tadulako.* 1 (1): 13-25 (Indonesia).

Ismaya, 2014. Biotechnology Artificial Insemination in Cattle and Buffaloes. Gadjah Mada University Press. Gadjah Mada University Press. Yogyakarta. (Indonesia).

Khan, R.U. 2011. Antioxidant and Poultry Semen Quality. *World's Poultry Science Journal*. 67 : 298-308.

Lubis, T.M. 2011. Sperm Motility Native Chicken in Diluent Coconut Water, Coconut Water and NaCl Physiological and NaCl Physiological at 25-29 °C. *Agripet*. 11 (2) : 45-50. (Indonesia).

Lukman H.Y., Busono, W., Wahyuningsih, S. and Suyadi, 2014. Sperm Motility and Viability after a-Tocopherol Dilution in Tris Aminomethane-Base Extender During Cold Storage in Bali Bull. *International Journal of ChemTech Research*. 6 (14) : 5726-5732.

Malecki, I. and Martin, G.B. 2002. Semen collection in the emu and ostrich. *Proceedings of the World Ostrich Congress. Warsaw, Poland*. 38-43.

Peters S.O., Shoyebo, O.D., Ilori, . B.M., Ozoje, M.O., Ikeobi, C.O.N. and Adebambo, O.A. 2008. Semen quality traits of seven strain of chickens raised in humid tropics. *International Journal of Poultry Science*. 7 : 949-953.

Ridwan dan Rusdin, 2008. Conservation Semen of Native Chicken Using Various diluents Against Fertility And Period Fertil of Sperm Post artificial insemination. *J. Agroland* 15 (1) : 63 -67. (Indonesia).

Rizal, M. dan Herdis, 2008. Artificial Insemination in Sheep. Penerbit Rineka Cipta. Jakarta (Indonesia).

Rizal, M. dan Herdis, 2010. Role of Antioxidants Improve quality of frozen Semen. *Wartazoa*. 20 (30) : 139-145. Indonesia).

Solihati, N., Pinnezak, A., Idi, R., Setiawan, R., Asmara, I.Y. and Sujana, B.I. 2006. The Storage Time Effect of The Local Chicken Chilled Semen at 5°C on Fertility and Fertile Period of Sperm. *Sperma. Jurnal Ilmu Ternak*. 6 (1) : 7-11 (Indonesia).

Surai, P.F., Fujihara,N., Speake, B.K., Brillard, J.P., Wishart, G.J. and Sparks, N.H.C. 2001. Polyunsaturated Fatty Acids, Lipid Peroxidation and Antioxidant Protection in Avian semen. Review. *Asian-Aust, J. Animal Science*. 14 (7) : 1024-1050.

Susilawati, T. 2011. *Spermatology*. UB Press. Malang. (Indonesia).

Thananurak, P., Sittikasamkit, C., Vongpralub, T. and Sakwiwatkul, K. 2015.Effects of Addition of Reduced Glutathione to Thawing media on Motility Parameters, Lipid Peroxidation and Fertility Rate in Frozen-Thawed Chicken Spermatozoa. *Khon Kaen Agr Journal*. 43 (Supll. 2): 98-102.

Triwulanningsih, E., Situmorang, P., Sugiarti,T., Sianturi, R.G. and Dan D.A. Kusumaningrum, 2003. Glutathione Addition Effect at Sperm Diluent Medium on the Quality of Chilled Semen .*JITV* .8 (2): 91-97 (Indonesia).

Wang, X., R.K Sharma., A. Gupta., V. George., A.J. Thomas., T. Falconie., and A. Agarwal. 2003. Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. *Fertility and Sterility.* 80 (Supllement 2) : 844-850.

Wiyanti, D.C., Isnaini dan, N. and Trisunuwati, P. 2013. Effect of Long Save Semen In Diluent NaCl Physiological At Room Temperature on the Quality of Sperm Native Chicken (*Gallus domesticus*). Journal of Veterinary Medicine.7 (1): 53-55 (Indonesia).

Zahariev, Z., Stefanof, R., Sabev, M., Miteva, K. and Nikolav, I. 2007. Cytochrome Affect Viability and Fertility of Bull Semen, am-Euras. *J. Agric and Environ*. 2 (1) : 48-50