Bali Medical Journal (Bali Med J) 2016, Volume 4, Number 3: 152- 156
P-ISSN.2089-1180, E-ISSN.2302-2914

Lactose-Astaxanthin Increases Green Jungle Fowl's Sperm Motility and Reduces Sperm DNA Fragmentation During 5º Celsius Storage

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Background: Good quality of semen is required for artificial insemination technology in ex-situ conservation efforts of green jungle fowl. This study was aimed to investigate semen quality of green jungle fowl during storage at 5ºC for 48 hours with the addition of combination lactose-astaxanthin in egg yolk phosphate dilution. **Method:** The semen used in the study was collected from eight healthy male green jungle fowls by using massage techniques. The semen quality was analyzed with macroscopic and microscopic examinations. The semen was diluted with egg yolk phosphate with the addition of 0.6% lactose, 0,004% astaxanthin and combination 0.6% Laktose-0,004% astaxanthin, and was stored at 5ºC for 48 hours. Following 48-hour treatment, the semen quality was evaluated based on its progressive motility, and DNA fragmentation. Data were firstly analyzed by using analysis of variance (ANOVA), and were then proceeded by using Duncan Multiple Range test. **Results:** The results showed that the progressive motilities of semen diluted in 0.6% lactose combined with astaxanthin 0.004% %, (79,66 ± 1.50%) was significantly higher than those diluted in 0.6% lactose (66,77 ± 2.16%,) and in astaxanthin 0.004% (68,11 ± 3.01 %). The DNA fragmentation of semen diluted inn 0.6% lactose combined with astaxanthin 0.004% %, (7,55 ± 1,66%) was significantly lower than those diluted in 0.6% lactose (12,33 ± 1,93%) and in astaxanthin 0.004% (13,55 ± 1,81%). **Conclusions:** In conclusion, the combination of l 0.6% lactose -astaxanthin 0.004% showed the best results for progressive motility, and DNA fragmentation.

Keywords: lactose, astaxanthin, green jungle fowl semen quality, storage at 5ºC

DOI:10.15562/bmj.v4i3.189

Cite This Article: Bebas, Wayan et al. Lactose-Astaxanthin Increases Green Jungle Fowl's Sperm Motility and Reduces Sperm DNA Fragmentation During 5o Celsius Storage. Bali Medical Journal4(3): 152-156.

INTRODUCTION

Green jungle fowls are now critically endangered germplasm in Indonesia as their number continuously declining. Artificial insemination technology has been proven to be able to help the breeding of endangered species through ex-situ conservation.[1,2] In the application of artificial insemination, the availability of semen with good quality is important. Therefore, semen needs to be treated properly by diluting it in appropriate dilution media and temperatures so that quality of the semen can be maintained for a certain time.

However, the process of cool storage semen can cause physical stress/cold shock and oxidative stress.[3,4]

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Cold shock can affect the structure and biochemical composition of sperms which can affect their function and ultimately leading to cell death.[5] Cold shock causes structural damage of the plasma membrane resulting in leakage of the membrane and enzymes out of spermatozoa cells. Enzymes such as the enzyme glucose-6-phosphatase, dehydrogenase, aspartate aminotransferase (Aspat), and the e ATPase sodium-potassium-linked pump (Na+/K+-ATPase).[6-10] Play a role in metabolic processes, and in plasma membrane proteins dislocation such groups glucose transporter (GLUT).[11] Cell membrane leakage affects cell function affecting motility, DNA damage and ultimately leading to cell death.

Cold storage also causes spermatozoa undergo oxidative stress.[3] Levels of Poly unsaturated fatty acid (PUFA) in the plasma membrane of poultry spermatozoa is commonly very high,[12] which is useful to preserve fluidity and flexibility membrane especially for motility and

fusion with the oocyte in the process of fertilization.[13] High level of PUFA in plasma membrane is very susceptible to peroxidation process which produces a molecule reactive oxygen species (ROS).[6,7,14] ROS also causes damage to DNA bases and phosphodiester bond.[15-17] The high ROS in spermatozoa causes the damage not only the plasma membrane but also DNA of the spermatozoa especially on the head of spermatozoa.[3] In some cases, ROS has been shown to cause damage to a single and double-stranded DNA in spermatozoa of infertile men, DNA fragmentation, and chromatin cross lingked.[1,11] This DNA damage triggers apoptosis which is also known as programmed cell death.[18]

To cope with physical stress and oxidative stress during cold storage, it is necessary to dilute an anti cold shock and antioxidants into diluent. Lactose is one of the ingredients that is capable of acting as anti-cold shock and also serves as a source of energy. While astaxanthin is a high-powered antioxidant that is expected to be able to cope with the process of peroxidation during the process of semen storage at cold temperatures.

METHODS

The research design used in this study was Randomized Posttest Only Factorial Design. Semen used was collected from eight healthy males green jungle fowlsby using massage techniques according to Burrows and Quinn method. Collected semen was evaluated macroscopically and microscopically. Good quality semen was diluted in phosphate yolk with the addition of 0.6% lactose, 0.004%, astaxanthin and 0.6% lactose combination 0.004%. astaxanthin as treatments. Each treatment was replicated nine times, and the semen was then stored at a temperature of 5^{o}C for 48 hours. After 48 hours of storage, semen from each treatment was evaluated for spermatozoa progressive motility, and DNA fragmentation.

Motility examination conducted by adding one drop (0.05 mL) semen on a glass slide at 37°C and adding another 2 drops of diluent phosphate yolk. Following a proper mixing, a drop of the mixture was put on a new glass slide. The slide was examined by microscope for sperm progressive motility. Sperm progressive motility was expressed as percentage of sperm with progressive motility.
Examination of DNA fragment performed by using TUNEL {Terminal-Deoxynucleotidyl-Transferase (TDT) mediated of labeled (X) deoxyuridine triphosphate nucleotides (X-dUTP) Nick-End-Labeling} (USA). TUNEL assay detected DNA fragmentation on single or double chain. DNA fragments were labeled at 3'-hydroxyl ends with dUTP-biotin.using TdT enzyme labeled DNA fragment at by adding dUTP coupled biotin. The dUTP=biotin was then detected by streptovidin – horse radish peroxidase (HRP) The data obtained were analyzed by applying analysis of variance (ANOVA), and proceeded by Duncan test.

RESULT

Macroscopic and microscopic profiles of semen collected from eight male green jungle fowls were shown in Table 1

Table 1. Macroscopic and microscopic profiles of semen collected from eight male green jungle fowls were shown in Table 1

Semen Quality	
Macroscopic examination	
Volume (ml)	0,60
Semen color	White
Viscosity Semen	Viscous
Odor	characteristic
Acidity/pH	7,2
Microscopic examination	2,5
Concentration (10^9/ml)	
Sperm Progressive (%)	89 P
abnormalities (%)	7,3
Mass Movement	++
Live spermatozoa (%)	92

Description: ++ = Mass motility good.
P = Progressive motility

The green jungle fowl semen used in the study was in good quality and worth for further processing. The semen concentration was 2.5 x 10^9 cells /ml with progressive motility of 89% and with abnormal spermatozoa of 7.3%. The mass movement scored at ++. Before treatment with several diluents, the semen was diluted to a concentration of 150.106 progressively motile spermatozoa cells per 0.2 ml.

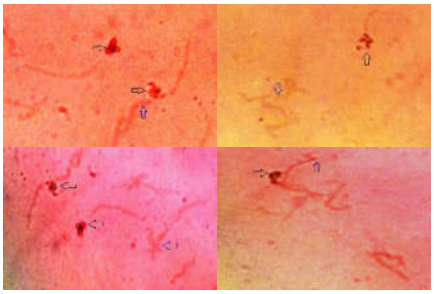
Data for spermatozoa progressive motility and DNA fragmentation of semen stored at 5°C for 48 hours are shown at Table 2.

Table 2. Progressive Motility and DNA Fragmentation of Green Jungle Fowl Sperms Stored at 5 ° C for 48 Hours.

Semen Quality	Treatment		
	T1	T2	T3
Progressive motility (%)	66,77 ± 2.16^{a}	68,11 ± 3.01^{a}	79,66 ± 1.50^{b}
DNA Fragment (%)	12,33 ± 1,93^{a}	13,55 ± 1,81^{a}	7,55 ± 1,66^{b}

Description: T1 = 0.6% lactose. T2 = 0.004% Astaxanthin. T3 = 0.6% Lactose Combination -Astaxanthin 0.004%. Different letters towards the line showed significant differences (P <0.05)

The microscopic examination of DNA fragmentation in the sperm of green jungle fowl is shown in Figure 1.



Description:
⇨ = DNA Fragmentation at the core of spermatozoa (1000x) using TUNEL Test
⇨ = Not Experiencing DNA Fragmentation

Figure 1. DNA Fragmentation of Green Jungle Fowl Sperm that Stored at 5°C for 48 Hours Using TUNEL Test.

DISCUSSION

Progressive motility

The percentages of progressively motile cells in 0.6% lactose (66,77 ± 2.16%), and in 0.004% astaxanthin (68,11 ± 3.01%) appeared to be suitable for use in artificial insemination as they were still in the level of the Indonesian National Standard (SNI) for viable semen used for insemination after undergoing a process of storage have to have progressive motility > 40%. The percentage of spermatozoa with progressive motility in combined 0.6% lactose -astaxanthin 0.004% (79.66 ± to 1.50%) was better than in0.6% lactose (66,77 ± 2.16%), and in 0.004% astaxanthin (68,11 ± 3.01%). Combined treatment of 0.6% lactose- 0.004% astaxanthin appeared to be able to give the maximum protection of the structure of the plasma and mitochondrial membranes as well as and other organelles of sperm cells against the physical stress due to the effects of cold shock and against oxidative stress caused by ROS attack

Lactose is a disaccharide carbohydrate consisting of two monosaccharide groups, I-e: one glucose and one galactose units. Plasma membrane of fowl spermatozoa contains carbohydrates binding with lipids (glycolipids) or proteins (glycoproteins) called sheath cells or glycocalyx.[19] Carbohydrates on plasma membranes like: sialic acid, mannose, glucose, and galactose.[20] have a very important biological functions to maintain a proper structural organization and biological specificity r on the surface of animal cells.[21]

Lactose also has the ability of replacing water molecules normally in the hydrated polar groups.[44] In other words lactose helps to stabilize cell plasma membrane during the critical temperature zone by altering the mechanical properties of the diluent through an increase in viscosity. Lactose can also interact directly with the center of phospholipid polar group during cooling and lowering bonding interactions of the van der Waals.

Lactose that consists of two monosaccharide units, glucose and galactose, can be metabolized by spermatozoa through glycolysis and/or Krebs cycle to produce energy in the form of adenosine triphosphate (ATP). Spermatozoa have metabolic features required for glycolysis, the citric acid cycle and oxidative phosphorylation in the mitochondria.[20] The energy produced by the metabolism of carbohydrates is in a form of ATP is then converted into adenosine diphosphate (ADP), and adenosine monophosphate (AMP),[21-24] which is then used for the movement and survival of spermatozoa. The addition of lactose in the diluents seems to be capable of protecting the plasma membrane fluidity and flexibility during cold storage.

Astaxanthin is an antioxidant with a specific molecular structure characterized by the presence of oxygen as a hydroxyl group (OH), and a carbonyl group (C = O) or a combination of both. The presence of hydroxyl and carbonyl functional groups in ketocarotenoid (polyene chain), creates a powerful antioxidant astaxanthin.[26] Its unique structure contains with two terminal rings that tend to be oriented at /near the surface of the membrane while the polyene chain function in the inner membrane. Thus, astaxanthin can be effective in preventing ROS at the surface of the membrane, while the polyene chain inhibits oxidative chain reaction in the inner membrane. Therefore, astaxanthin is an excellent antioxidant that protect all cellular components from degenerative damage and ROS attack.[20]

Astaxanthin defense strategy against ROS is based its ability to outage physical processes, where immediate transfer of energy between the two molecules. As the energy of singlet oxygen molecules is transferred to the carotenoid molecule, ground state triplet oxygen and triplet exits carotenoids are then gained. Excess of energy from the excited molecule is the transferred through the energy releasing mechanism.[25] Mechanism reaction of carotenoids as a quencher of singlet oxygen are as follows:

1. $O2 + 1Carotenoid \rightarrow 3O2 + 3Carotenoid^*$

2. Energy is released through rotation and vibration interactions between carotenoid triplet with solvent to restore its original carotenoids.[26]
3. Carotenoid* → $_1$Carotenoid +thermal energy.

Astaxanthin can protect essential biological functions of sperm such as protecting and fighting peroxidation of lipid membrane such as PUFA and proteins, DNA damage, the effect of UV light, and also plays an important role in the immune response.[16] The addition of combined 0.6% lactose-0.004 % astaxanthin 0.004% which has good protection on progressive motility on chicken semen has also been reported by Al-Daraji (2011). In which best result was obtained by adding green tea infusion as an antioxidant. Additionally, a research conducted by Al-Daraji (2012), was done by adding various extracts of garlic and the best concentration was 2 ml/100 ml of diluent, produced 65% progressive motility. Research result done by Donoghue and Donoghue (1997), was by adding vitamin C (Tempo) and the best concentration was 0.156 μM resulted in progressive motility of 68.8 $\pm$ 3.9%.[28]

DNA Fragmentation

The addition of 0.6% lactose, 0.004% astaxanthin and 0.6% lactose combined with 0.04% astaxanthin during cold storage, resulted in DNA fragmentation: 12,33 $\pm$ 1,93%; 13,55 $\pm$ 1,81%; Dan 7,55 $\pm$ 1,66% respectively. The least DNA fragmentation was found at the treatment group with addition of 0.6% lactose combined with astaxanthin 0.004%

Storage of semen at cold temperatures can cause physical stress/cold shock and oxidative stress (OS).[15,36] OS is the main cause of DNA demage.[9,28] as DNA bases and phosphodiester bonds are highly susceptible to peroxidation. OS is formed because ROS and antioxidants scavengers that neutralize ROS are not balanced. Incidence of DNA fragmentation positively correlated ($r = 0.4$; $p = 0.02$) with the ROS forming.[9] Spermatozoa that were exposed to ROS in vitro are DNA damage in the form of modified bases, loss of bases of DNA structure, deletions, changes in the structure of DNA, cross linkage between DNA and chromosomal alterations.[28] OS on DNA can increase DNA fragmentation that causing an increase in the content of mutagenesis of 8-hydroxy-2-deoxyguanosin (8-OH-2-dG),[18] while to Ball (2008), reported DNA fragmentation is not only caused by OS but also osmotic stress. According Siudzinska and Lukaszewcz (2008), semen storage at cold temperature impacts to damage the cell membrane such at the destruction of ATP-ase-linked sodium-potassium pump (Na^+/K^+-ATPase).[8,17] Pump Na^+/K^+ -ATPase function for maintain the concentration gradient of Na^+ and K^+ in the cell. In order to maintain cell volume with a regulated solute in the cell and minimize the effect of osmosis resulting the cells undergo swelling or shrinking.[11] The addition of combination 0.6% lactose-astaxanthin 0.004% is very effectively to cope with DNA fragmentation. This is due to the lactose role in maintaining plasma membrane fluidity and flexibility so that the role of the plasma membrane remains existed. Astaxanthin with hydroxyl and carbonyl functional group in ketocarotenoid (polyene chain), make astaxanthin as a strong antioxidant,[26] in preventing ROS with extinction physics process.[30] Astaxanthin can prevent or reduce the oxidized guanosine.[21] Astaxanthin as a powerful antioxidant has demonstrated a beneficial effect in various diseases associated with oxidative damage, such as: hypertension,[26] obesities,[27] muscular degenerations, and cancer.[30]

CONCLUSION

It can be concluded that the addition of combination 0.6% lactose -astaxanthin 0.004% give the best results in progressive motility and DNA fragmentation on green jungle fowl semen that storage at a temperature of 5°C for 48 hours.

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