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HSP70 Expression and DNA Molecular Changes of Baluran Bull on Post Thawing Sperm

Keywords: HSP70, DNA, frozen semen, Limousin bull infertility

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Research Articel

HSP70 Expression and DNA Molecular Changes of Baluran Bull on Post Thawing Sperm

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Abstract

Cross breeding program by using artificial insemination technique with frozen semen aimed to increase the productivity of beef cattle, but there were some reports of infertility cases in crossbreed cows. Based on its reason was need designed research to identify molecular changes in post thawing of sperm DNA of Baluran bull. This study reconstructed the freezing semen Limousin bull named Baluran, accompanied by the identification of HSP70 expression, PCR and nucleotide sequencing of sperm post thawing. The result of the study indicated that post-thawing sperm quality of Baluran bull comparison with established standards, there is increased expression of HSP70 compared to fresh semen. Nucleotide sequence of fresh sperm exactly same with Gene Bank data, but there was a 12 nucleotide changes in post-thawing sperm compared to fresh sperm in Gene Bank data, ie in seven thymine, four guanine, and one andonecytocine. Based on these findings it could be concluded that there were molecular changes in the DNA of sperm Baluran bull post thawing.

Keywords : HSP70, DNA, frozen semen, Limousin bull infertility

1. Introduction

The increase of beef cow productivity through cross breeding program by employing artificial insemination technique is aimed to combine two or more beneficial properties which initially exist in two kinds of cows into one crossbreed cow. Farmers favor Limousin crossbreed cow as it gains lots of weights in a day, it cost higher when sold alive so that its market price is higher than local cow (Sutarno and Setyawan, 2015). Furthermore, artificial insemination program which is run intensively can reveal some cases regarding to infertility in artificial insemination acceptor. Several reports showed that the efficiency of female crossbreed cow productivity is lower than local cows (Desinawati and Isnaini, 2010; Dwiyanto and Inounu, 2009; Endrawati et al. 2010; Ihsan, 2010; Nuryadi and Wahjuningsih, 2011).

In the production of frozen semen, many unidentified factors can trigger changes of spermatozoa both cellular and molecular. The possible problem that might occur during the production of frozen semen is spermatozoa cold-shock phenomenon caused by intracellular changes resulted by ice crystal forming. In processed of frozen semen, ice crystal forming caused electrolit and soluble materials accumulation in cells that can harm of sperm intracellular segment. It is difficult to detect intracellular damage occurring and it raises the chance the spermatozoa to die. This causes the amino acid structure to change and it will affect

sperm vitality (Hafez and Hafez, 2013). Cryoprotectant is expected to be able to surmount stress occurring as the effect of frost-bonding process that instigates the increase of the number of protein which undergo denaturation or damaged protein, thus it increases heat shock protein (HSP) as a form of protection to damages cell. Extracellular and intracellular cryoprotectant is capable to protect cells from extreme temperature changes. The use of ethylene glycol dan sucrose can protect cells from cold shock and the raise of HSP70. Heat shock protein is a compound that possesses biochemical activity as chaperon molecule (HSP40, HSP60, HSP70, and HSP90), it is to restore the protein that experience of misfolding, unfloding, or abnormal folded protein, synthesis of protein, protein transport and translocation process and to prevent protein aggregation by partaking in the synthesis of protein (Zhang et al., 2016). Intracellular damages will trigger DNA fragmentation as well. DNA fragmentation in sprematozoa occurs not at the frost-bonding process, but it occurs at thawing process (Men et al., 2003; Said et al., 2010). Damages in DNA feared cause mutation of protein structure of a cell, that affects sperm viability and motility. But, if the DNA damages of sperm at thawing process do not affect the viability, motility, and integrity of spermatozoa membrane, so the spermatozoa stays fertile, afford to fertilize ovum and develop as fetus and is born safely, therefore it is essential to observe of phenotype.

Based on above matters, reconstruction of frozen semen process was run as the source of 50% genetic properties of Baluran female crossbreed cows. Bio molecular analysis towards HSP70 expression as a protection against apoptosis and the profile of spermatozoa's DNA post-thawing is compared to novel spermatozoa, thus the possibility of molecular changes in spermatozoa properties bearer to its offspring can be identified.

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2. Materials and Methods

The cattles used in this research are male Limousin cows named Baluran; it is nine years old (born in December 17, 2005), its ear number is 80518, its registration number is BLM011INAJ051832031, certified and its semen could be proceed to form frozen semen in frost-bonding semen laboratory BIB Faculty of Veterinary Medicine, Universitas Airlangga which is located in Taman Ternak Pendidikan Universitas Airlangga, Gresik, Indonesia.

The method of freezing Baluran's semen was carried out in accordance with the exact procedure or standard operational prosedur (SOP) based on regulation of the Director General of Animal Husbandry of Republic Indonesia, number 12207/ HK.060/ F / 12/ 2007 concerning technical guidelines for the production and distribution of frozen semen.

The examination of HSP70 was conducted by immunohistochemical staining and was aimed to calculate the total of spermatozoa which possess positive immunoreactive towards HSP70. The data of immunoreactive cell in this examination is quantitative data which is retrieved by calculating the total of immunoreactive cells found in every 100 spermatozoa cells (Fuchs and Auer, 2010). Immunoreactive spermatozoa will be coloured chromogen brown up to blackish, while negative immunoreactive cells will be not coloured. The examination used regular luminescence microscope Nikon H600L which is equipped with digital camera DS Fi2 300 megapixels and image processing software and cell count Nikon Image System.

In this study, the sample from frozen semen process was drawn randomly as many as 24 batches from the whole process, while for the sperm's nucleotide examination, the sample was drawn randomly as many as 10 batches. Sequence

data of post-thawing sperm's nucleotide was compared to fresh sperm and Gen Bank data to describe the possibility of changes that occur

3. Results

Like the other processes of frozen semen, there was a decrease in parameter of post-thawing frozen semen quality compared to fresh semen; it was the viability from 89.67 ± 4.03% to 60.00 ± 3.30%, the motility from 76.87 ± 4.90% to 50.42 ± 4.87%, and the integrity of sperm membrane from 65.88 ± 5.82% to 29.00 ± 3.55% (Table 1). The value of post thawing motility in this research is 50.42 ± 4.87%. It was considered fine, standard minimal as the percentage sperm motility before freezing is 60% and post-thawing is 40% (Komariah et al,2013).

Based on the result of this study, it was indicated that the percentage of HSP70 extraction in fresh semen group was different to frozen semen group. There was an increase of HSP70 extraction percentage in frozen semen group as much as 30% compared to fresh semen group which is extracted only as much as 10% (Table 2). Spermatozoa which show positive immunoreactive extraction against HSP70 looked brown (Fuchs and Auer, 2010) in its head and neckline (Figure 1).

Characterization by PCR shows that nucleotide band as large as 427 bp is thinner for post-thawing sperm comparised than the fresh sperm (Figure 2). This is in line with absorbance value, wherein this situation occurs because at post-thawing sperm have undertaken dissolving by added diluter as many as ten or fifteen times the fresh semen volume. It is spotted out that ten samples of sperm of fresh semen that retrieved from Limousin cow named Baluran retains the same nucleotide sequence as other Limousin cow in Gene Bank. It is indicated that male cow named Baluran which is brought into this study is still stable as in line with the certificate. In ten post-thawing sperm samples of Limousin cow show the similar nucleotide sequence.

In post-thawing sperm, there are differences of 12 nucleotide alkali compared to fresh sperm nucleotide sequence. Those changes are included seven Thymine nucleotide (T), four Guanine nucleotide (G), and one Cytocine nucleotide (C). According to amino acid coding nucleotide triplet, those changes are followed by the changes of nine amino acid, but three of them do not undertake any changes (Figure 3).

In nucleotide number 101, T switches over to G, so that the amino acid coding triplet of nucleotide switches over form TTG to TGG, it results in the change of amino acid and hypothetically changes from leucine to tryptophan. In nucleotide number 112, G switches over to A, and in nucleotide number 113, G switches over to C, thus the amino acid coding triplet of nucleotide changes from GGT to ACT, it results in the change of amino acid and hypothetically changes form threonine to glycine. In nucleotide number 239, T switches over to C, thus the amino acid coding triplet of nucleotide changes from CTG to CCG, it results in the change of amino acid and hypothetically changes form leucine to proline. In nucleotide number 264, T switches over to A, therefore the amino acid coding triplet of nucleotide changes from TTT to TTA, it results in the change of amino acid and hypothetically changes form phenylalanine to leucine.

In nucleotide number 268, G switches over to C, therefore the amino acid coding triplet of nucleotide changes from GGG to CGG, it results in the change of amino acid and hypothetically changes form glycine to arginine. In nucleotide number 346, T switches over to C, therefore the amino acid coding triplet of nucleotide changes from TAG to CAG, it results in the change of amino acid and hypothetically changes form leucine to glutamine. In nucleotide number 418 and 419, respectively, T and C switches over to

A and T, therefore the amino acid coding triplet of nucleotide changes from TCC to ATC, it results in the change of amino acid and hypothetically changes form serine to isoleucine (Table 3)

4. Discussion

By the time this study was conducted, this male cow Baluran was nine years old. The sperm motility post-thawing of male cow in aged 3, 8, 9, and 11 years respectively are 47,8±1,8; 43,8±2,1;47, 0±2,3; 46,8±1,2% (Aminasari, 2009). Frozen semen will get damaged by 40% and to produce supreme quality of frozen semen to be used as the inseminated spermatozoa, dissolvent substances to can persist the quality of sperm (viability, motility, and membrane integrity) is essentially needed during either freezing or dilution process (Paulenzet et al., 2002). Membrane integrity damage is initiates the opening of extrinsic factor access against DNA, thus it is possible that DNA fragmentation takes place (Hafez and Hafez, 2013).

In the process of frozen semen, the use of cryoprotectant is expected to protect spermatozoa from cold shock and increases the level of HSP70. HSP70 retains bio chemical activity to restore protein which undergo misfolding, unfloding, or abnormal folded protein, synthesis of protein, protein transport and translocation process and to prevent aggregation (Zhang et al., 2016). Therefore, the existence of HSP70 is considered as the indication of spermatozoa protection as the result of freezing process.

This provides clues that freezing process causes sperm cell to get stressful. Stress as the result of freezing process triggers changes of protein structure and its role inside cell. Cell will extract some vital protein in order to protect its self through restoring and degrading the protein which is already damaged and it goes by intracellularly in a particular trail. The several studies reported that there was found a group of microscopic-molecule-size-protein which is called heat stress protein or widely known as heat shock protein (HSP) taking role actively in the process of preserving the cells to subsist (Kacimi et a., 2010). The result of this study shows that there is an increase of extraction in one of HSP (HSP70) in the group of frozen semen.

Genetically, 50% properties of female cow inherited from its parents (local cows) dan the rest 50% inherited from frozen semen which is inseminated. If we see from the genotype, 50% of DNA from the parents is noe affected by external factors during ovulation process, fertilization process to the forming of fetus, being born and growing as adult female cows. Nevertheless, the spermatozoa used for insemination undergoes additional diluter, frezzing, and thawing before it fertilize the ovum. Consequently, follow-up study is required to find whether or not there is any change towards the sequence of post-thawing sperm's nucleotide acid which is used for insemination in this study.

There are three alteration of nukleotida, but it does not affect to the alteration of amino acid which is coded by those three nukleotida. On 118 nukleotida, it is changed from G to C so that the amino acid synthesis coding triplet of nuklotida are changed from GAG to CAG but they two code the amino acid glutamin as well. On 149 nukleotida, it is changed from T to C so the the amino acid synthesis coding triplet of nukleotida are changed from CAT to CAC but they two code histidin amino acid as well. While on 161 nukleotida, it is changed from T to C so the the amino acid synthesis coding triplet of nukleotida are changed from CTT to CTC but they two code leusin amino acid as well.

There are two factors that cause damage in the DNA of spermatozoa. They are strees oksidatif (OS) and apoptosis. OS is known as the unbalance condition between the production of reactive species oxygen (ROS) by spermatozoa and leukosit, and the capacity of seminalis

antioksidan plasma. The over nicotinamide adenine diphosphate hydrogen (NADH) which is produced through glucose 6 phosphate dehydrogenase on the sitoplasma then stimulate the peroduction of ROS. Apoptosis is the crucial component on spermatozoatogenesis which function to control the amount of germinal cell that turned into spermatozoatozoa so that it is placed on the point which can be supported by sertoli cell. In addition, apoptosis can cause the low quality of semen on the concentration of low spermatozoa, defected sperm and even the damage of DNA (Cakici and Akoz, 2017).

The integrity of paternal DNA is important for further embryo growth. The level of DNA damage is correlated with the reduction of embryo growth and the damage of DNA that may cause serious fertility. The broken sperm DNA does not disrupt the bond of sperm with pelusida zone on the ovum, while the fertilization and the embryo split will be normal. The emergence of infertility which is caused by the sperm DNA diversion is not at the level of fertilization but it exists at the initial DNA extraction on the embryo growth.the damage of DNA does not affect to the sperm fertility since the membrane and sperm organel are integrally functioned. But the damage of DNA causes the decreasing of embryo growth after the genom embrionik extraction. Therefore, in one way, alive sperm and important motile to be able to fertilize the ovum, but it does not work on the process occurred after conception.

On the other hand, DNA of sperm DNA does not work on the fertilization process but it is crucially proceed on the embryo growth from the embryonic DNA extraction after the first split of zygote. Thus, the unity of spermatozoa membrane and DNA are the two important components on the sub-fertility and infertility [16]. The damages in DNA of sperm do not affect the ability of sperm either to fertilize oocytes or to undergo 2-3 times of split, but it can blockade blastocysts forming apoptosisly [6], therefore pregnancy does not occur. The damages of DNA does not trigger the death of embryo, because intial embryo stadium can take a part in restoring the damage of sperm DNA, thus the effect of sperm fragmentation in new-formed individual depends on the combined effect of sperm, chromatin damages, and the capacity of oocytes to restore it (Cakici and Akoz, 2017).

In the process of artificial insemination, after the semen is sprayed intra cervically, spermatozoa undergoes capacitacy right away, and its motility moves to the area where the fertilization takes place. When fertilization takes place, the head of spermatozoa which bring 1n genetic material from male cow will meet 1n genetic material from the female cow to form zygote with 2n genetic material which consists of 60 chromosomes. Based on the combination of that genetic material, several processes entangled protein synthesis occurs to let the zygote grows into embryo and to become fetus which inherit that parents' properties. Phenotypic property is the interaction of genotypic and the environment. The changes of spermatozoa nucleotide sequence can trigger amino acid coding triplet of nucleotide, so that it can interfere molecular reaction and physiological process that implicates those amino acid changes. The abnormality of chromosome can cause infertility and repeat breeder cow (RBC) syndrome (Silva and Gadella, 2006).

Acknowledgments

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Conflict of Interest

There is no conflict of interest

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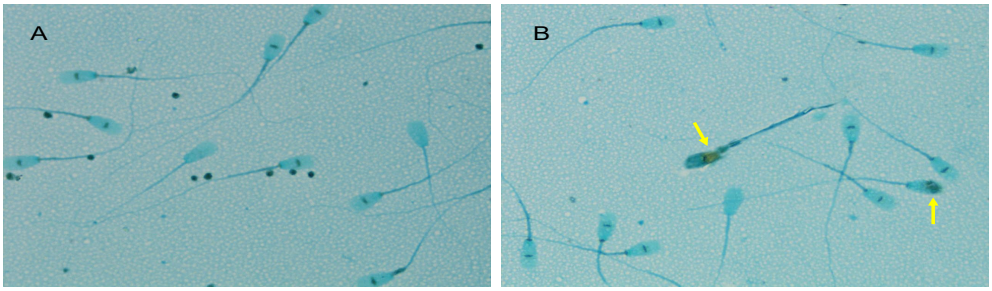


Figure 1. Negative extraction of HSP 70 (Figure A) dan positive (Figure B) at the head and neckline of spermatozoa (arrow) (HE staining. Zooming 1000x; Nikon H600L microscope; DS Fi2 300 megapixel camera).

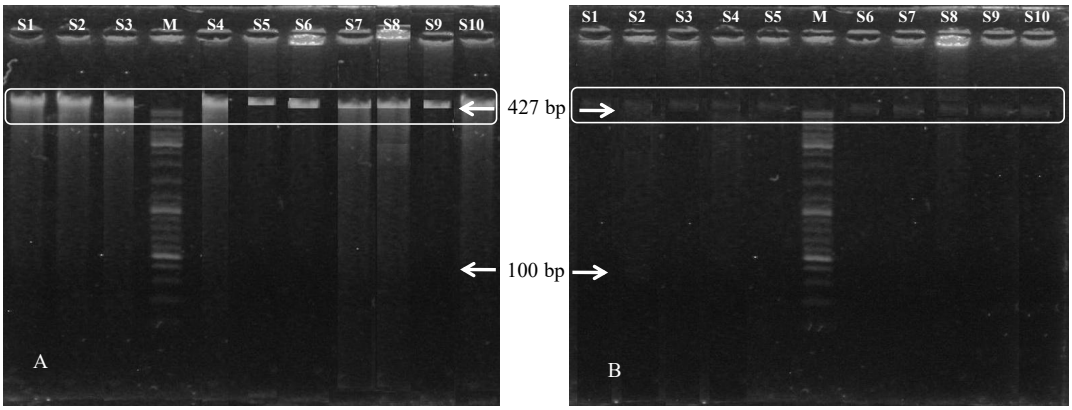
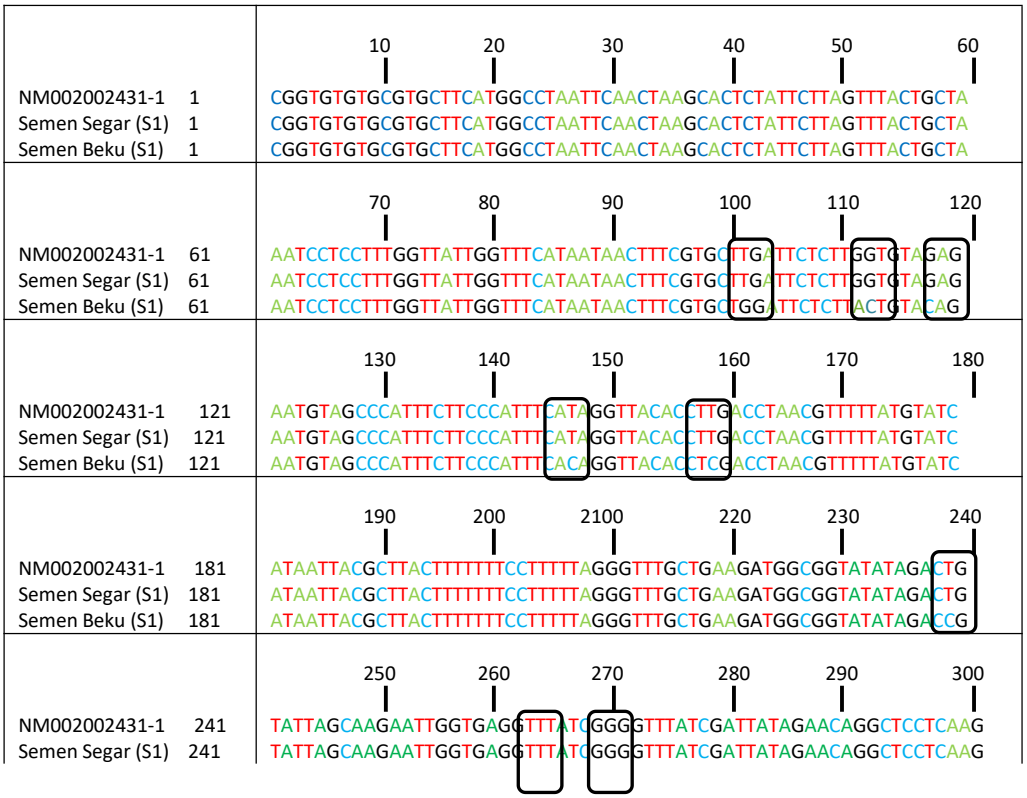


Figure 2. Analysis of coding gene of Limousin beef cattle by PCR before freezing visualized by electrophoresis in 1% agarose gel ethidium bromide staining. The result shows that cDNA thread is as large as 427 bp., A: fresh sperm, B: post thawing sperm, S1-10 = Samples 1-10, M = Marker



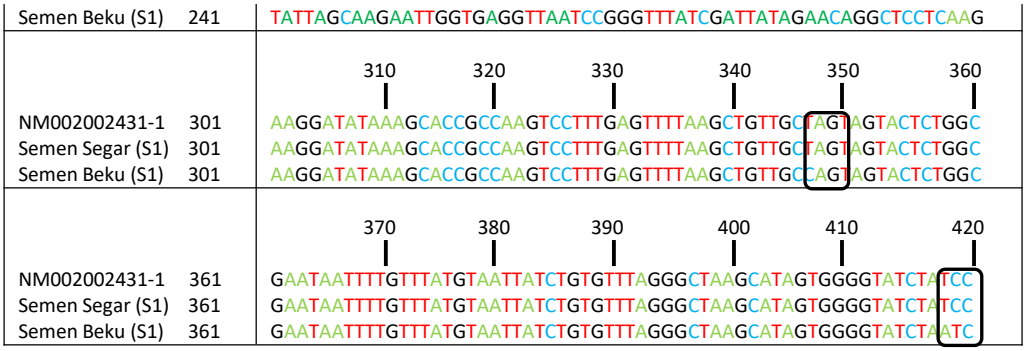


Figure 3. Molecular changes in sequencing analysis report and the changes of amino acid coding triplet codes (inside the boxes) from post thawing spermatozoa of Baluran cow by using multiple alignment. NM002002431-1 : data of nucleotide sequence from Gene Bank

Table 1. The examination of viability, motility and membrane integrity of spermatozoa of (%) Baluran Limousin cow before and after freezing

	Viability	Motility	Membrane Integrity
Fresh Semen	89,67 ± 4,03	76,87 ± 4,90	65,88 ± 5,82
Frozen Semen (post thawing)	60,00 ± 3,30	50,42 ± 4,87	29,00 ± 3,55

Table 2 The result of staining HSP70 immunocytochemical protein and motility of frozen and fresh semen from Baluran Limousin cow which is used for artificial to female cow in East Java

Semen	Positive Immunoreactive (%)	Motility (%)
Frozen semen	30	45
Fresh semen	10	80

Table 3. The changes of nucleotide alkali, amino acid codes and amino of frozen semen (FS) compared to fresh semen and data from Gene Bank (GB)

No	No of Nucleotide Sequence	Basa Nucleotide		Amino Acid Code		Change of Amino Acid		Explanation
		GB	FS	GB	FS	GB	FS	
1	101	T	G	TTG	TGG	Leusin	Triptofan	fluctuate
2	112	G	A	GGT	ACT	Threonin	Glisin	Fluctuate
3	113	G	C					
4	118	G	C	GAG	CAG	Glutamin	Glutamin	Static

5	149	T	C	CAT	CAC	Histidin	Histidin	Static
6	161	T	C	CTT	CTC	Leusin	Leusin	Static
7	239	T	C	CTG	CCG	Leusin	Prolin	Fluctuate
8	264	T	A	TTT	TTA	Phenilalanin	Leusin	Fluctuate
9	268	G	C	GGG	CGG	Glisin	Arginin	Fluctuate
10	346	T	C	TAG	CAG	Leusin	Glutamin	Fluctuate
11	418	T	A	TCC	ATC	Serin	Isoleusin	Fluctuate
12	419	C	T					

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Effect of Heat Shock Protein (HSP) in Post Thawing Baluran Bull Semen

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Abstract

Artificial insemination with frozen semen of Limousin bulls was evaluated for the post thawing changes in quality characteristics in semen and their effect on the sperm viability motility and spermatozoa membrane integrity. Besides the frost-bonding effect on the production of heat shock protein (HSP) and their protecting nature against the extreme temperature changes on the extra and intracellular integrity of spermatozoa were evaluated. Twenty four semen samples randomly drawn were subjected to freezing as per regulation of the Director General of Animal Husbandry, Indonesia. The frozen semen after thawing showed decreasing viability from 89.67±4.03% to 60.00±3.30%; motility from 76.87±4.90% to 50.42±4.87% and sperm membrane integrity from 65.88±5.82% to 29.00±3.55%. These values were in accordance with that of minimum percentage of motility of 40 percent.

Key words : Frozen semen, thawing quality, Limousin bulls.

Beef cattle breeding favours artificial insemination with semen from superior bulls to produce off springs which can attain higher weight at marketing age (Sutarno and Setyawan, 2015). The production of frozen semen can trigger changes in

spermatozoa both in cellular and molecular level. One such problem that might occur during semen freezing is cold-shock resulting in ice crystal formations, electrolyte soluble materials accumulation in cells, which could damage the intracellular segment of spermatozoa. This effect could cause the structural changes in the amino acid of spermatozoa, which again affects the sperm viability (Hafez and Hafez, 2013). The effect of frost-bonding process instigates the protein denaturation and increase the production of heat shock protein (HSP). The use of ethylene glycol and sucrose can protect cells from cold shock and increase the production of HSP70 a variant of heat shock protein. This will protect the damaged cells from extreme temperature fluctuations. The damages during thawing process can affect the sperm viability, motility, membrane integrity and the per cent fertile of spermatozoa. Hence the study of freezing the Baluran bull semen was conducted to assess their effect on the above characteristics.

Materials and Methods

Limousin bulls (Baluran) was utilized in the study of post freezing analysis on the changes in the semen quality parameters. The study was conducted at the frost-bonding semen laboratory BIB Faculty of Veterinary Medicine located at Taman Ternak Pendidikan UniversitasAirlangga, Gresik, Indonesia.

The freezing of Baluran bull semen was carried out as per the standard operational procedure (SOP) of the Director General of Animal Husbandry of Republic Indonesia (2007) guidelines for production and distribution of frozen semen.

The examination of HSP70 was done to calculate the total spermatozoa possessing positive immunoreaction as per (Fuchs and Auer, 2010).

Immunoreactive spermatozoa will initiate production of coloured chromogen which changes from brown to blackish, while negative immunoreative cells will not exhibit coloured reaction. The examination was conducted with luminescence microscope Nikon H600L. Twenty four frozen semen samples randomly drawn were used for the quality assessment in comparison with that of fresh semen to assess the changes that might occur during the freezing and thawing process.

Results and Discussion

The semen quality changes are presented in the Table I.

Table I. The quality characterization of Baluran Limousin bulls fresh semen and thawed frozen semen and their HSP70 immunoreactivity percentage.

	Quality parameters			
Semen samples	**Viability**	**Motility**	**Membrane Integrity**	**Immunocytochemical protein HSP70**
Fresh	89.67±4.03	76.87±4.90	65.88±5.82	10
Frozen	60.00± 3.30	50.42±4.87	29.00±3.55	30

There was a decrease in the per cent viability from 89.67 to 60.00, motility from 76.87 to 50.42 and integrity of spermatozoa membrane from 65.88 to 29.00. The post thawing motility per cent was 50.42 which is considered as acceptable as minimum sperm motility as standard (Komariah *et al*. 2013).

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To: ivj83@yahoo.com

Date: Sunday, June 24, 2018 at 10:25 PM GMT+7

Dear Editor Indian Veterinary Journal

Please find attached article after revision and Author comments .

Sincerely

Corresponding Author,
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3. If there is any postal pin code no. or zip code no. for Surabaya, it may be furnished in the address of the places where the work was carried out.
4. Since there were lot of mistakes in your original article, a sample revised article is enclosed for your reference. Revised article and a soft copy may be submitted as short communication for further action.

AUTHOR COMMENTS

1. The PCR study is not acceptable as per referees comment as it appear to have plagiarism from other source → Yes I agree.
2. IVJ format. Kacimi et al. year is 2000, NOT 2010.
3. postal pin code no. or zip code no. for Surabaya = 60115 → I have added it .
4. Revised article and a soft copy will be submitted as short communication for further action.

Thank you for your attention and cooperation

Effect of Heat Shock Protein (HSP) in Post Thawing Baluran Bull Semen

Trilas Sardjito, Pudji Srianto, Chairul Anwar Nidom,

Imam Mustofa and Erma Safitri[1]

Department of Reproduction Veteriner, Faculty of Veterinary Medicine,

Universitas Airlangga, Surabaya, Indonesia, 60115

[1]*Corresponding author* : Email : rma_fispro@yahoo.com

Abstract

Artificial insemination with frozen semen of Limousin bulls was evaluated for the post thawing changes in quality characteristics in semen and their effect on the sperm viability, motility and spermatozoa membrane integrity. Besides the frost-bonding effect on the production of heat shock protein (HSP) and their protecting nature against the extreme temperature changes on the extra and intracellular integrity of spermatozoa were evaluated. Twenty four semen samples randomly drawn were subjected to freezing as per regulation of the Director General of Animal Husbandry, Indonesia. The frozen semen after thawing showed decreasing viability from 89.67±4.03% to 60.00±3.30%; motility from 76.87±4.90% to 50.42±4.87% and sperm membrane integrity from 65.88±5.82% to 29.00±3.55%. These values were in accordance with that of minimum percentage of motility of 40 percent.

Key words : Frozen semen, thawing quality, Limousin bulls.

Introduction

Beef cattle breeding favours artificial insemination with semen from superior bulls to produce off springs which can attain higher weight at marketing age (Sutarno

and Setyawan, 2015). The production of frozen semen can trigger changes in spermatozoa both in cellular and molecular level. One such problem that might occur during semen freezing is cold-shock resulting in ice crystal formations, electrolyte soluble materials accumulation in cells, which could damage the intracellular segment of spermatozoa. This effect could cause the structural changes in the amino acid of spermatozoa, which again affects the sperm viability (Hafez and Hafez, 2013). The effect of frost-bonding process instigates the protein denaturation and increase the production of heat shock protein (HSP). The use of ethylene glycol and sucrose can protect cells from cold shock and increase the production of HSP70 a variant of heat shock protein. This will protect the damaged cells from extreme temperature fluctuations. The damages during thawing process can affect the sperm viability, motility, membrane integrity and the per cent fertile of spermatozoa. Hence the study of freezing the Baluran bull semen was conducted to assess their effect on the above characteristics.

Materials and Methods

Limousin bulls (Baluran) was utilized in the study of post freezing analysis on the changes in the semen quality parameters. The study was conducted at the frost-bonding semen laboratory BIB Faculty of Veterinary Medicine located at Taman Ternak Pendidikan UniversitasAirlangga, Gresik, Indonesia.

The freezing of Baluran bull semen was carried out as per the standard operational procedure (SOP) of the Director General of Animal Husbandry of Republic Indonesia (2007) guidelines for production and distribution of frozen semen.

The examination of HSP70 was done to calculate the total spermatozoa possessing positive immunoreaction as per (Fuchs and Auer, 2010). Immunoreactive spermatozoa will initiate production of coloured chromogen which changes from brown to blackish, while negative immunoreative cells will not exhibit coloured reaction. The examination was conducted with luminescence microscope Nikon H600L. Twenty four frozen semen samples randomly drawn were used for the quality assessment in comparison with that of fresh semen to assess the changes that might occur during the freezing and thawing process.

Results and Discussion

The semen quality changes are presented in the Table I.

Table I. The quality characterization of Baluran Limousin bulls fresh semen and thawed frozen semen and their HSP70 immunoreactivity percentage.

	Quality parameters			
Semen samples	**Viability**	**Motility**	**Membrane Integrity**	**Immunocytochemical protein HSP70**
Fresh	89.67±4.03	76.87±4.90	65.88±5.82	10
Frozen	60.00± 3.30	50.42±4.87	29.00±3.55	30

There was a decrease in the per cent viability from 89.67 to 60.00, motility from 76.87 to 50.42 and integrity of spermatozoa membrane from 65.88 to 29.00. The post thawing motility per cent was 50.42 which is considered as acceptable as minimum sperm motility as standard (Komariah *et al*. 2013).

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