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**SUPPLEMENTATION OF SPECIFIC PROTEINS IN THE SEMINAL PLASMA
FROZEN SEMEN DILUTER MEDIAON THE PLASMA MEMBRANE INTACT,
THE EXPRESSION OF CASPASE AND MALONDIALDEHYDE POST THAWING**

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Abstract

Background: The quality of frozen semen after thawing is one of factors in artificial in semination. Cryopreservation affects the post-thawed sperm quality. The seminal plasmadiary bull's contains high concentration of specific proteinosteopontin. Our hypothesis is that osteopontin maintain sperm quality during cryopreservation.

Aim :The aim was to investigate osteopontin does improve the quality of frozen semen as appears from increased The plasma membraneintact (PMI) , decreased expression of caspase and Malondialdehyde (MDA)

Method : isolation, identification and protein specification of osteopontin from dairy bullsemen. using SDS-PAGE method indicated that there were some tapes showing differentmolecular weight from several samples. SDS-PAGE result from protein identification wascontinued with protein identification using Western Blot technique. The effect of osteopontinon the quality of frozen semen post thawing by the expression ofcaspasewithimmunocytochemistry techniques andmeasurementof MDA

Result: of the first phase was determined that molecular weight protein specification of osteopontin is 56 kDa. Osteopontin concentration from electroelution samples were analyzed using Nano drop method, and it showed the protein concentrations as avaregely 180 µg /ml. result of the second phase indicated that the addition of osteopontin can improve semen quality through increased The plasma membraneintact (PMI), decreased of expression caspase 9 and MDA

Conclusion : Addition of osteopontin may improve semen quality through increased The plasma membraneintact (PMI) decreasedofexpressioncaspase9 and MDA

Key words: Spesifik Protein, Friesian Holstein diary bull, seminal plasma, PMI, MDA, Caspase 9

INTRODUCTION

On the freezing until thawing, problems often arise causing cold-shock (cold shock) due to changes in temperature. Cold-shock is one of the causes of oxidative stress due to increased Reactive Oxygen Species (ROS) excess, thus stimulating lipid peroxidation (Lenzi et al., 2002 and Kankofer et al., 2005). According to Bilodeau et al. (2001), Chatterjee et al. (2001) and Lenzi et al. (2002), mammals spermatozoa are rich in unsaturated fatty acids and are thus highly sensitive to ROS. The key mechanism of ROS has been implicated in the plasma membrane and mitochondrial damage. Disorders of the mitochondrial membrane can result in the release of cytochrome c which would trigger the activation of caspase and end in apoptotic cell death (Green and Reed, 1998).

According to Sudjana (2008) ROS excess must be eliminated so as not to lead to cell damage. The mechanism for eliminating cells through a ROS scavenger enzyme (anti-ROS) or so-called antioxidants, such as superoxide dismutase (SOD) and catalase. Superoxide dismutase is an enzyme that transforms superoxide radicals that are dangerous to the cell into hydrogen peroxide, while catalase is an enzyme that converts hydrogen peroxide to water (H₂O) and oxygen (O₂). If the scavenger enzyme is impaired, the ROS will be greater than the anti-ROS, thus causing damage and behavioral changes. Reactive Oxygen Species (ROS) can break the double bonds of unsaturated fatty acids that make up the system membrane of sperm cells, forming a substance called malondialdehyde (MDA).

Osteopontin is a specific protein in the seminal plasma, acidic, rich in aspartic acid, glutamic acid and serine (Sorenson and Petersen, 1994 cited Maura et al., 2006). Glutamic acid is a compound of glutathione which is a primary antioxidant. Wijaya (1996) quoted Triwulanningsih et al. (2003) stated that glutathione is a tripeptide containing three amino acids: glutamic acid, glycine and cysteine, which are primary antioxidants that work by preventing the formation of new free radicals. Results of research by Kaeoket et al. (2008) reported a decrease of glutathione can maintain the quality of spermatozoa on the freezing and post-thawing.

MATERIALS AND METHODS

Effect of osteopontin on semen

Preparation of semen freezing medium (diluter), Semen was mixed with semen freezing medium adding 0,5,10,15 or 20 osteopontin per 50 million spermatozoa to determine the effect of osteopontin on quality of frozen semen post thawing to examine the expression of caspase and MDA in the post-thawing spermatozoa dairy osteopontin FH after the addition of semen to the freezing medium through immunocytochemistry techniques

Examination of caspase 9 expression through immunocytochemistry technique

Expression of caspase in spermatozoa dairy cows can be seen after the addition of various doses of osteopontin treatment technique used immunocytochemistry with monoclonal antibody caspase. Furthermore colored using immunocytochemistry techniques. Examination of the amount of caspase expression in dairy cows performed spermatozoa after freezing (post-thawing). Sperm thawing straw first and then dropped into the glass object, flooded with another glass object with an angle of 45°. Preparations were then washed in PBS pH 7.4 for 3 x 5 minutes. Furthermore, immersed in 3% hydrogen peroxide (in Water) for 5-10 minutes. Washed in PBS pH 7.4 for 3 x 5 minutes. Caspase was added primary antibody (Bioworld, no paint. BS-2741) for 1 hour at room temperature, then washed with PBS pH 7.4 for 3 x 5 minutes. Added anti-rabbit secondary antibody labeled with biotin-IgG for 1 hour at room temperature. Washed in PBS pH 7.4 for 3 x 5 minutes. Then added SA-HRP (Horseradish Peroxidase Streptavidin-) for 30-60 min at room temperature after it was washed in PBS pH 7.4 for 3 x 5 minutes, then added 3,3-diaminobenzidine tetrahydrochloride chromogen (DAB) for 10-20 minutes at room temperature then washed in distilled water for 3 x 5 minutes. Do counterstain with hematoxylin for 5 minutes at room temperature, then washed with distilled water for 3 x 5 minutes. Further mounting with entellan. Observation using an optical microscope with 400x magnification (Nurhidayat, 2002).

Measurement of levels MDA (Malondialdehyde) Postthawing the sperm Dairy Cattle After addition of Osteopontin in Semen Freezing Media

A total of 100 mL of sperm suspension is separated from the protein by the addition of 100 mL of 20% trichloroacetic acid and vortex for 30 seconds, then add 250 mL of 1N HCl, 100 mL of 1% thiobarbituric sodium and distilled water until the final volume to 1 mL (450 µl). Then heated in a water bath at a 100°C for 20 minutes and centrifugation at a speed of 3500 rpm for 10 minutes. Seanyak 800 mL of the supernatant is inserted in another tube and add distilled water to a final volume of 2 mL. Uptake color read using a spectrophotometer at a wavelength of 529 nm. Rate of MDA is done by converting the value of the absorbance measurement results with the standard curve value standard pure MDA in different concentrations. Furthermore, the result of multiplying the value of the gold standard curve, multiplied by the dilution factor used. MDA levels measured by the MDA nmol/g sperm suspension of spermatozoa or spermatozoa concentration per mL.

Membrane Integrity Examination Presentation Postthawing Spermatozoa Dairy Cattle In FHafter addition of Osteopontin in Semen Freezing Media via Hypoosmotic Swelling Test Method (HOS Test)

Examination of cell membrane integrity of spermatozoa presentation was conducted by hypoosmotic swelling test (HOST) developed by Jayendra et al. (1984). Suspensions of spermatozoa from frozen semen originating from cows that have been added to various concentrations of osteopontin (T0, T1, T2, T3, T4) is taken as 0.1 ml and add 9.9 ml of 0.032 M hypoosmotic (prepared from 7.35 g of sodium citrate 2H₂O, 13.52 g fructose dissolved in 1 liter of distilled water). Furthermore incubated for 1 hour in a CO₂ incubator at 37 ° C. Then a thin cover made preparations by mixing a drop of the above solution with one drop of eosin and observed with a light microscope 400x magnification. Spermatozoa have plasma membrane integrity intact tail section visible presence of swelling followed the tail rotates with a radiant light colors, while spermatozoa with damaged plasma membrane is characterized by the absence of swelling of the head with a straight tail.

RESULT

Osteopontin treatment of the MDA show, P0 significantly different from T1 ($p > 0.05$), and highly significant to T2, T3 and T4 ($p < 0.01$). The same thing was seen in T1 to T2, T3 and T4 which shows the difference in MDA were significantly ($p < 0.01$). Osteopontin treatment at T2 to T3 and T4 showed significant differences against ($p < 0.01$), T3 to T4 osteopontin treatments showed no significant difference in MDA ($p > 0.05$). Osteopontin treatment against caspase shows that T0 to T1 was not significantly different ($p > 0.05$) but significantly different with T2, T3 and T4 ($p < 0.01$). T1 is not significantly different from T2 and T3 ($p > 0.05$), but significantly different with T4 ($p < 0.05$). T2 is not significantly different from the T3 and T4 ($p > 0.05$). The same thing was also shown by T3 and T4 ($p > 0.05$). More result on level MDA and expression caspase 9 osteopontin treatment are presented on table 1, examination result expression caspase 9 are presented in figure 1. PMI on osteopontin treatment T1 highly significant both to T2, T3 and T4 ($p < 0.01$). T2 to T3 or T4 did not show significant differences ($p > 0.05$). Similarly, between T3 and T4 showed no difference PMI significantly ($p > 0.05$). The result of the calculation of osteopontin treatment of the PMI can be seen in Table 1. The results of the examination PMI spermatozoa presented in the figure 2

Table 1. Osteopontin treatment on level MDA, expression caspase 9 and MPI

Treatment	MDA (mean± sd)	Caspase 9 (mean± sd)	MPI (mean± sd)
T0	21,7277 ^a ±1,78050	22,0563 ^a ±3,80200	44,8725 ^a
T1	17,7468 ^b ±1,20673	18,2050 ^{ab} ±4,24476	47,3925 ^a
T2	13,8944 ^c ±0,97365	14,4213 ^{bc} ±3,95086	53,4450 ^b
T3	11,4639 ^d ±0,58024	13,5750 ^{bc} ±2,89198	54,8363 ^b
T4	9,6305 ^e ±0,76980	12,1637 ^c ±2,37793	56,5388 ^b



Figure 1. Caspase-9 expression of sperm frozen postthawing. (1000X)

(A) spermatozoa expressing caspase-9 appears the brownish color, especially in the core area of spermatozoa (red arrows)

(B) spermatozoa do not express caspase-9 (yellow arrow).

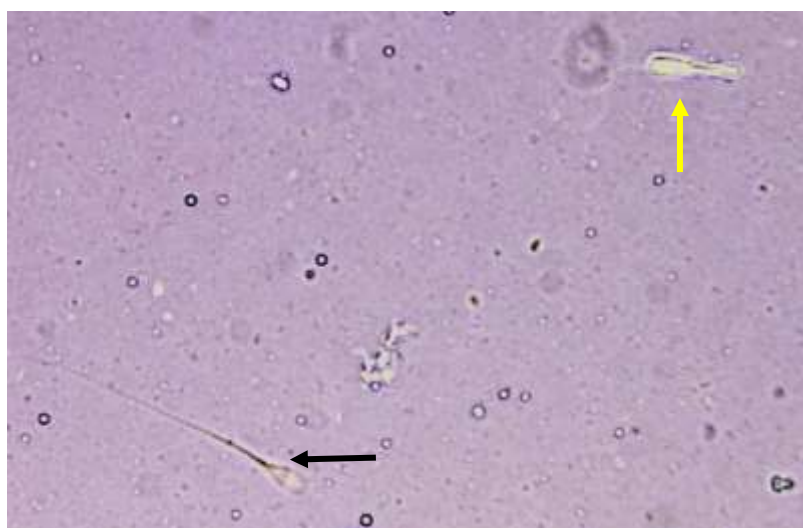


Figure 2. PM examination results sperm postthawing of frozen semen.

A) damaged sperm plasma membrane (black arrow)

B) sperm with intact plasma membrane (yellow arrow)

Discussion

Effect of osteopontin levels of MDA spermatozoa after thawing frozen dairy cows. Freezing is one of the most important techniques in the manufacturing process of frozen semen. However, during the cryopreservation temperature change and extreme osmolality. In general, semen freezing problem revolves around the two main issues, namely cold-shock and intracellular changes due to discharge, which is associated with the formation of ice crystals (Mumu, 2009).

The results of this study indicate that among the group without addition of osteopontin significantly different from the group of 5 µg osteopontin administration, and highly significant to the group of osteopontin administration of 10 µg, 15 µg and 20 µg. The same thing was seen in the group of osteopontin administration of 5 µg to 10 µg, 15 µg and 20 µg of demonstrating the very real differences in MDA. Group of osteopontin administration of 10 µg against the administration of 15 µg and 20 µg showed significant differences, osteopontin administration of 15 µg group against osteopontin administration of 20 µg group showed no significant difference in decreasing MDA.

This proves that the addition of osteopontin with a dose of 15 µg an optimal dose in inhibiting the increase in ROS as evidenced by decreased levels of MDA in spermatozoa post thawing frozen. According to Bansal and Bilaspuri (2010) Mechanism spermatozoa by ROS damage is induced lipid membrane in spermatozoa and react with the formation of lipid peroxidation (LPO). Susceptibility to cold temperatures associated with the ratio of unsaturated acid content is high (Poly-unsaturated Fatty Acid/PUFA) than saturated fats contained in the sperm plasma membrane. Cholesterol is one of unsaturated fatty acids that make up the sperm plasma membrane. Osteopontin added having the amino acid sequence contains one of which serves to maintain the condition redox of oxidation of proteins through the reversible oxidation of the amino acid Nordberg and Arner (2001). Terminal Sequence Analysis of osteopontin produces a sequence of 15 amino acids (VKPXSSGXSEEKQLN) with 85% homologous to known cattle deoxyribonuclease (DNase) I-like protein. (Cancel, et al., 1997). According Feradis (2009) is the most important component of the cell membrane phospholipids, glycolipids and cholesterol. The first two components containing polyunsaturated fatty acids that are highly susceptible to free radical attack, especially hydroxyl radical (OH^{*}). These hydroxyl radicals can cause a chain reaction known as lipid peroxidation. According to Bucak et al (2010) sperm cells contain a high content of polyunsaturated fatty acids, making the membrane more susceptible to damage peroxidation. Spermatozoa membrane is expected to be

the main target or cold shock freezing damage to cells at the processing of frozen semen, cryopreservation has been known to generate Reactive Oxygen Species (ROS). ROS is one of oxidants or free radicals, in which the main properties of the free radical itself is to have one or more unpaired electrons in their outermost track. This has resulted in ROS into components that are reactive with certain substances. At normal levels in the sperm, ROS are very helpful in the process of hyperactivation, capacitation, acrosome reaction, and binding of the zone (zone bindings) (Kodama et al, 1996; de Lamirande et al, 1997).

Some studies show that ROS play an important role in fertility/infertility in sperm. When the balance between ROS production and detoxification by antioxidants is interrupted, excess ROS trigger oxidative stress. ROS (one of which is H_2O_2) is known to function as a hold motility and sperm block oxidative metabolism. Also, ROS lower the penetration of oocytes by sperm and sperm-oocyte fusion blockade in a mouse through a mechanism that involves oxidation process (Mammoto et al, 1996 in Bailey, 2011). DNA damage in sperm by ROS have also been reported to have serious consequences for the development of post fertilization (Aitken et al, 1998, in Awda et al, 2009).

Malondialdehyde (MDA) is one group of aldehydes produced due to the peroxidation of fatty acids Polyunsaturated that has more double bonds such as linoleic acid, arachidonic acid and docosahexanoic acid (DHA) membranes, therefore the increase in MDA in suspension is generally used as one indicator for membrane lipid peroxidation (Alvarez and Storey, 1995; Halliwell and Gutteridge, 1999). As it is known that high accumulation of ROS production together with low content of antioxidants in the seminal plasma ROS formed will cause more reactive. If the capacity and antioxidant response in the seminal plasma is not able to neutralize ROS are formed as quickly and as big as ROS is formed, then the balance between ROS production and antioxidant is disturbed. Sequence Arg-Gly-Asp on osteopontin interacts with cell surface receptors, such as integrins. osteopontin is known to perform adhesion between cells, increasing communication between the extracellular matrix, reduce the production of ROS and nitric oxide in tissues that suffered injury, as well as the change in intracellular calcium levels (Johnson et al., 2003). According Baldi et al (1996) and Amin (2000) to high intracellular calcium concentration will increase the activity of the enzyme adenylate cyclase, thereby increasing cAMP concentration ensuing phosphorylation of enzymes leading to high motility. Osteopontin as with vitamin E as an antioxidant can reduce levels of MDA (Agarwal, A. 2005). Osteopontin containing gamma glutamyl acid can act as a scavenger that inhibits lipid peroxidation reaction so that the membrane can lower the levels

ofMDA. A similar mechanism is also found in the activity of glutathione inhibit membrane lipid peroxidation (Barycki, 2008)

Effect of osteopontin on the expression of caspase 9 in spermatozoa after thawing frozen dairy cows. The results showed that without the addition of osteopontin against caspase 9 was not significantly different from the group of osteopontin administration 5 ug but highly significant to the group giving 10ug, 15ug and 20ug. 5ug osteopontin administration group was not significantly different from the group of osteopontin administration of 10ug and 15ug, but significantly different from the group of osteopontin administration of 20ug. Osteopontin administration of 10ug group was not significantly different from the group of osteopontin administration of 20ug group. The same thing is also shown by the group of osteopontin administration of 15ug and 20ug osteopontin administration groups were not significantly different. This result means a dose of 10ug osteopontin administration alone get the expression of caspase 9 reduced the percentage of spermatozoa.

The results of this research were supported by the results of research conducted by Martin et al. (2006) who observed that the process of cryopreservation or thawing causes a decrease in viability as associated with the incidence of apoptosis. It is based on the change in the mitochondrial membrane, causing an increase in caspase-9 and Bax, Bcl-2 but factor in the studies were not detected.

This study was not detected on caspase 3, in line with Martin et al. (2006), the expression of caspase-3 was not detected in the study. In addition, studies have been carried out by Hendricks and Hansen (2009) observed in the apoptotic cell death pathways post ejaculated spermatozoa in the two species, namely horses and cows. Both the cow and horse spermatozoa, pro active caspase-9 that caspase 3 is detected but not detected. Through the integrin receptor, osteopontin able to activate NF- κ B is associated with the expression of various genes that encode proteins pro-apoptotic and anti-apoptotic (Saile et al., 2001).

Effect of osteopontin on the plasma membrane intact (PMI) of the sperm post thawing frozen dairy cows. Both the plasma membrane and mitochondrial membrane of sperm cattle susceptible to the influence of cryopreservation (O'Connell et al., 2002). The main influence on the cryopreservation of sperm cells is a decrease in motility and vitality, permeability changes and changes in the membrane lipid components. The main influence on the cryopreservation of sperm cells is a decrease in motility and vitality, permeability changes and changes in the membrane lipid components. The onset of lipid peroxidation during semen freezing process affects the cell membrane damage in spermatozoa.

Physical damage of which can be either plasma damage and membrane acrossome (Ismaya, 2009) MPU osteopontin treatment to show that the group without giving osteopontin against osteopontin administration of 5 ug group did not show significant differences but without giving osteopontin group against group of osteopontin administration of 10 ug, 15 ug and 20 ug showed a very real difference. Percentage of osteopontin administration MPU group 5 highly significant that both the groups of osteopontin administration of 10 ug, 15 as well as in the group of osteopontin administration of 20 ug. Group of osteopontin administration of 10 ug P2 against 15 ug or 20 ug did not show significant differences. Similarly, among the group of osteopontin administration of 15 mg and 20 ug osteopontin administration groups showed no significant difference in apoptosis. The results of this study demonstrate that administration of a dose of 5 ug osteopontin has not been able to menstabilize sperm membrane from around spermatozoa and prove the addition of osteopontin with a dose of 15 ug an optimal dose in reducing the percentage of apoptosis in the process of freezing sperm.

The plasma membrane is the cell wall membrane which controls the exit and entry of some substances that are required in the process of metabolism and activity of living cells. The plasma membrane is composed of protein, carbohydrates and fat that can act as a receptor for a particular compound. Plasma membrane intact spermatozoa which serves as a protective overall cell survival spermatozoa. In addition, the integrity of the sperm membrane also acts as a protector of cellular organelles of mechanical or chemical damage, filter for the exchange intra- and extracellular compounds are retained in the metabolic processes (Garner and Hafez, 2000). Membrane integrity of spermatozoa is also applied in the evaluation of the quality of spermatozoa in the cement industry of frozen (Kennedy and Sutovsky, 2011).

The membrane consists of biomolecular lipid layer with proteins inserted in it or attached to one surface of the membrane. Integral membrane proteins embedded in the lipid layer and strong. Most of these proteins are fully stretched and double layer called transmembrane proteins, while others are embedded in the outer layer or double layer of lipids. Peripheral proteins bound loosely on the internal surface of the membrane. Many of the proteins and lipids that have oligosaccharide chains exposed out (Murray and Ganner, 2001).

Osteopontin is an extracellular matrix glycoprotein that is secreted into the seminal plasma and liquid accessory glands (Moura et al., 2006; Moura et al., 2007). The composition of osteopontin glycoprotein associated with the effect on sperm membrane stabilization. It is also related to the interaction with the fat in the form of lipoproteins, causing the membrane more flexible is not easily fragile. Ties between osteopontin, glucose and

fat can cause the particles between the membrane is collected, hence a density of membrane components so that more stable in the process of cooling, freezing and thawing again after freezing, the stability of the membrane of spermatozoa, the metabolism is running normally, the function of spermatozoa is better. The results are consistent with research conducted by Suprayogi (2013) which uses FAA containing glycoproteins. , Showed that the addition of osteopontin is effective in protecting spermatozoa in sperm freezing until it is thawing

CONCLUSION

Addition of osteopontin may improve semen quality through increased of the plasma membrane intact , decreased of expression caspase 9 and MDA

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