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THE EFFECT OF OSTEOPONTIN IN SEMEN FREEZING PROCESS ON THE QUALITY OF HOLSTEIN FRIESIAN DAIRY BULL FROZEN SEMEN.

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Abstract

Background : The quality of frozen semen after thawing is one of factors in artificial insemination. Cryopreservation affects the post-thawed sperm quality. The seminal plasma diary bull's contains high concentration of osteopontin. Our hypothesis is that osteopontin maintain sperm quality during cryopreservation.
Aim : The first aim was isolation, identification and protein specification of osteopontin from dairy bull semen. The second aim was to investigate osteopontin does improve the quality of frozen semen as appears from increased expression of HSP 70 drecreased apoptotic expression and increased viability and motility.
Method : isolation, identification and protein specification of osteopontin from dairy bull semen. using SDS-PAGE method indicated that there were some tapes showing different molecular weight from several samples. SDS-PAGE result from protein identification was continued with protein identification using Western Blot technique. The effect of osteopontin on the quality of frozen semen post thawing by the expression of HSP 70 with immunocytochemistry techniques. The expression of apoptotic spermatozoa by Tunnel Assay. The viability spermatozoa using negrosin eosin staining and by assesing motility of spermatozoa with direct microscopy.
Result of the first phase was determined that molecular weight 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 the increase of HSP70 resulting in a decrease in apopotosis. The result of all causes increased motility mechanisms, either directly or indirectly based on viability.
Conclusion : Addition of osteopontin may improve semen quality through increased HSP70 resulting in a decrease in viability and motility of apoptosis that can be maintained.

Key words: Osteopontin, Friesian Holstein diary bull, seminal plasma, post- thawed sperm quality.

INTRODUCTION

Freezing semen is intended to support the success of artificial insemination (AI) in livestock, especially dairy cows. One of the factors for the success of the AI program is the quality of the frozen semen used. At moment , especially generates pregnancy rates below 60% (Hendri, 2010). while figures Services per conception (S / C ratio) is high, about 2.7 to 2.8 (Susilowati, 2000)., Since 2005-2009, the S / C ratio has decreased to 1.9 but is still above the international standard of 1.65.

Dispite investigations done during decades still remained on the same constraints that sperm viability after thawing back remained low and limited to about 50% (Kankofter et al. 2005 and Andrabi 2009) . In the process of thawing semen, temperature changes cause the so called cold shock stimulate lipid peroxidation (Lenzi et al., 2002 and Kankofter et al., 2005). Mammalian spermatozoa are rich in unsaturated fatty acids of which the double bonds highly sensitive to Reactive Oxygen Species (ROS) (Bilodeau et al. 2001 and Chatterjee et al.2001). ROS damage the plasma membrane and mitochondrial. Disruption of the mitochondrial membrane results in the release of cytochrome c leading by caspase activation to apoptotic cell death (Green and Reed, 1998).

ROS are normally produced by spermatozoa, but when produced in excess will harm the spermatozoa. To protecte themselves the cells respond with production of heat shock protein (HSP). HSP has a protective role in protein homeostasis mechanisms by assisting the process of folding, transporting and assembling the protein in the cytoplasm, mitochondria and endoplasmic reticulum (Jeong et al, 2009)

Osteopontin is one of a group of proteins that can be found in large quantities in the seminal plasma. Osteopontin has a molecular weight of 55 kDa and is secreted by a gland ampulla. The presence of osteopontin in seminal plasma is closely related to the fertility of male animals (Cancel et al., 1997; Rodriguez et al., 2000).

We investigated wheter addition of osteopontin to frozen semen does improve the quality of the semen after thawing as assessed by increased expression of HSP 70, decreased apoptotic expression and increased viability and motility.

MATERIALS AND METHODS

Isolation osteopontin

Proteinwere extracted from seminal plasma osteopontin semen dairy cows by centrifugation and sonication, molecular weight of osteopontin by sodium dodecyl-

polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by Western blot using monoclonal antibodies against osteopontin (COSMO BIO Co., LTD, Cat no. LSL-LB-4225). Isolation of osteopontin in seminal plasma from fresh semen semen FH dairy cows with electro elution method; Calculation results osteopontin levels elution using Nanodrop. This assay was needed for research on second stage.

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 HSP 70 in the post-thawing spermatozoa dairy osteopontin FH after the addition of semen to the freezing medium through immunocytochemistry techniques. To examine the expression of apoptotic spermatozoa post-thawing on dairy cows FH after the addition of osteopontin to the media by the method of freezing semen Tunnel Assay using Apoptag Detection Kit (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, no paint S7101). To examine the percentage of spermatozoa viability post thawing in dairy cows osteopontin FH after the addition of semen to the freezing medium through negrosin eosin staining. To examine the percentage of spermatozoa motility basis microscope

Examination of HSP 70 expression through immunocytochemistry technique

Expression of HSP70 in spermatozoa dairy cows can be seen after the addition of various doses of osteopontin treatment technique used immunocytochemistry with monoclonal antibody HSP 70. Furthermore colored using immunocytochemistry techniques. Examination of the amount of HSP 70 expression in dairy cows performed spermatozoa after freezing (post-thawing). Sperm dithawing straw first and then dropped into the glass object, floaded with another glass object with an angle of 450. Preparations were then washed in PBS pH 7.4 for 3 x 5 minutes. Furthermore, immersed in 3% hydrogen peroxide (in DI Water) for 5-10 minutes. Washed in PBS pH 7.4 for 3 x 5 minutes. HSP 70 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 Strepavidin-) 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).

Examination Percentage Apoptosis Post thawing through Tunnel Assay Method Using Apoptag Detection Kit

The percentage of apoptosis in dairy cattle spermatozoa after addition of osteopontin in the post-thawing semen freezing medium can be determined through the method of using Apoptag Tunnel Detection Assay Kit (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, no paint S7101).

Examination Percentage Viability Post thawing through Negrosin Eosin Staining

Spermatozoa viability checks done by making preparations commentator. 10 mL suspension of semen dropped on the tip of a glass object, then drops dye eosin-negrosin mixed until homogenous with other glass objects attached to the ends of the semen mixture, then the magnification.position of the taper angle oblique thrust along the glass objects that have been prepared to obtain a layer of semen that has been dyed as thin as possible. Furthermore aerated to dry. Calculations performed using a light microscope 400x Spermatozoa with the head of spermatozoa which is colorless life, being pink is dead spermatozoa (Partodihardjo, 1992).

Post thawing motility examination percentage basis Microscope

Motility examination consists of a qualitative and quantitative examination. Ten microliters of the suspension of cement in each treatment group to the dripped glass objects contained in the center of the indentation. then covered with a cover glass and motility were observed by light microscopy magnification 400x. Quantitative examination of sperm motility is determined by level of progressive movement forward. The percentage is calculated based on the average movement of sperm motility percentage for all of the calculated field of view (Tuty, 2004).

Statistical Analysis

The design used in the study was a completely randomized design (CRD). All data normality test with Kolmogorof-Smirnov and Shapiro-Wilk. HSP 70 , apoptotic The design used in the study was a completely randomized design (CRD). All data normality test with Kolmogorof-Smirnov and Shapiro-Wilk. HSP 70 ,apoptotic expression and viability was analyzed using Analysis of Variant (ANOVA) followed, if there is a difference with 5% LSD (Least Significant Difference). For data that is not normal motiltas using Kruskal Wallis test, if there is a difference followed by Man Whitney U test (Steel and Torrie, 1995).

RESULT

Identification of Seminal Plasma Protein Osteopontin Cement Dairy Cattle FH with SDS-PAGE method. SDS-PAGE results are protein ribbons that appear shown in Figure 1.

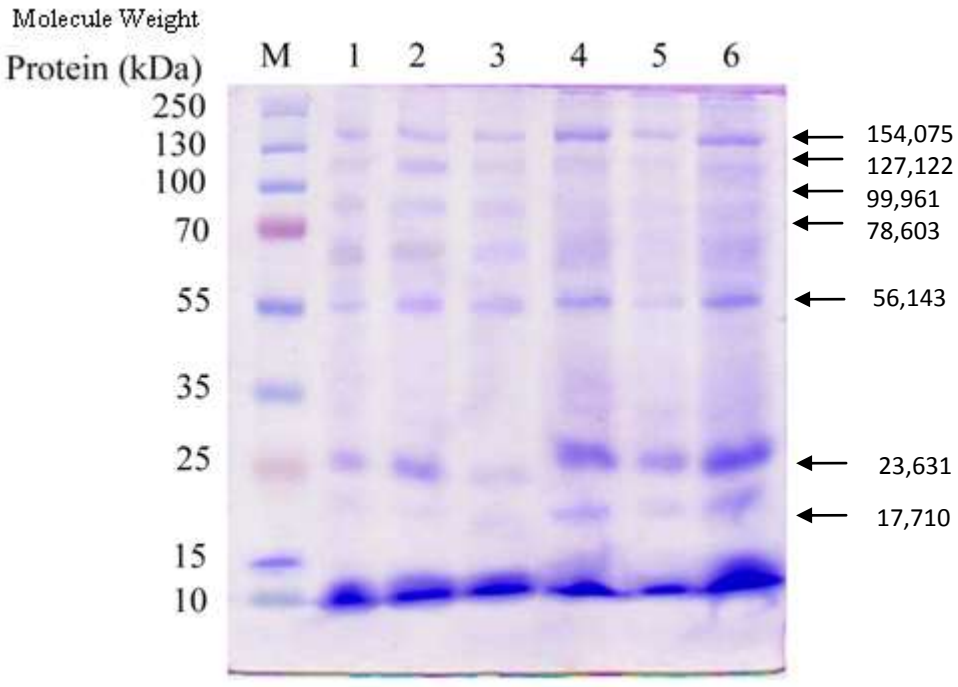

Figure 1. Results Identification ribbons protein isolated from seminal plasma FH semen dairy cows by SDS-PAGE

Identification of Seminal Plasma Protein Osteopontin FH Dairy Cattle by Western Blotting

Figure 2. Results of Western Blotting Analysis of osteopontin isolates with a molecular weight of 56 kDa that reacted with the monoclonal antibody anti-Osteopontin

Isolation of Seminal Plasma Osteopontin by electroelution method

Results of electroelution is a liquid containing osteopontin protein with a molecular weight of 56 kDa. Protein level Measurement Results Elution with Nano Drop

Table 1. Seminal Plasma Levels of Osteopontin in Dairy Cattle FH ug / ml per sample elution.

elution to	**Level of Osteopontin (µg/ml** sample elution**)**
1	130
2	100
3	230
4	200
5	200
6	220
Avarage	180

Effect of osteopontin on HSP 70, apoptotic, viability and motility of spermatozoa. Mechanism of action of osteopontin on the quality of spermatozoa

Effect of osteopontin treatment against HSP 70 are presented in Table 1, whereas the expression of HSP 70 is presented in Figure 3 As shown in Table 1, T0 , T1 was not significantly different ($p > 0.05$) but significantly different from P2 ($p < 0.05$) while the T3 and T4 showed a highly significant difference ($p < 0.01$). T1, T2 significantly different ($p < 0.05$) and highly significant with T3 and T4 ($p < 0.01$). T2 to T3 was not significantly different ($p > 0.05$) but significantly different from the T4 ($p < 0.05$). Not significantly different between T3 and T4.

Treatment of osteopontin in apototic show that T0, T1 was not significantly different ($p > 0.05$) but significantly different from T2 ($p < 0.05$). T3 and P4 treatment against ostopontin at P0 showed a highly significant difference ($p < 0.01$). T1 ,T2 compared to aposptosis osteopontin treatment showed no significant differences ($p > 0.05$), but becomes highly significant when compared to T3 or T4, T1 ($p < 0.01$). Treatment of osteopontin in T2 with T3 significantly different ($p < 0.05$) and highly significant with P4 ($p < 0.01$), whereas T3 to T4 showed no significant differences ($p > 0.05$) . More results osteopontin treatment to apoptosis are presented in Table 1. Expression of apoptotic spermatozoa are presented in Figure 4

Spermatozoa viability count results from application of osteopontin in detail is presented in Table 1. As seen in Table 1, T0 ,T1 was not significantly different ($p > 0.05$) but significantly different T2, T3 or T4 ($p < 0.01$). T1 to T2 also showed no significant difference in viability ($p > 0.05$), but when compared to T3 or T4 showed a highly significant difference ($p < 0.01$). The same thing happens between T2 to T3 or P4 showed differences in viability were highly significant ($p < 0.01$). T3 to T4 treatment on viability showed no significant differences ($p > 0.05$). Examination Results live and dead spermatozoa are presented in Figure 5

Treatment osteopontin motility showed T0 to T1 was not significantly different ($p > 0.05$). T0 than T2, T3 or T4 showed motility were highly significant ($p < 0.01$). T1 than T3 and T4 also showed highly significant differences in motility ($p < 0.01$). Similarly, between T2 to T3 and T4 showed a highly significant difference ($p < 0.01$), Similarly, between T2 to T3 and T4 showed a highly significant difference ($p < 0.01$), whereas T3 to T4 showed no

significant differences in motility (p> 0.05). More results on motility osteopontin treatment are presented in Table 2.

Table 2 . Osteopontin Treatment on Expression HSP 70, Apoptotic , viability and motility

Treatment	HSP 70 (mean± sd)	Apoptotic (mean± sd)	Viability (mean± sd)	Motility (mean± sd)
T0	23,6413^{a} ± 4,55165	6,3588^{a} ± 0,82067	56,4813^{a} ± 2,23466	44,3588^{a} ± 3,35379
T1	23,7950^{a} ± 3,76490	6,0163^{ab} ±0,85162	59,2387^{ab} ± 3,05029	45,6963^{a} ± 4,45068
T2	28,8413^{b}± 4,42051	4,9725^{b}± 0,99394	62,0963^{b}±2,325510	52,3950^{b}±2,38664
T3	33,3825^{bc}± 3,15892	3,5125^{c}± 0,88978	68,5663^{c}± 3,50911	59,3413^{c}±2,15252
T4	34,3375^{c}± 2,34775	2,2563^{c}± 0,82097	72,0038^{c}± 2,0776	61,2463^{c}±1,76289

Different superscripts in the same column differed significantly (p <0.01)

Figure 3 . HSP 70 expression of sperm frozen semen post thawing (1000 X)

- spermatozoa do not express HSP 70 (black arrow).
- spermatozoa that express HSP 70 appears the color brownish especially on the acrosome (head) spermatozoa (arrows red).

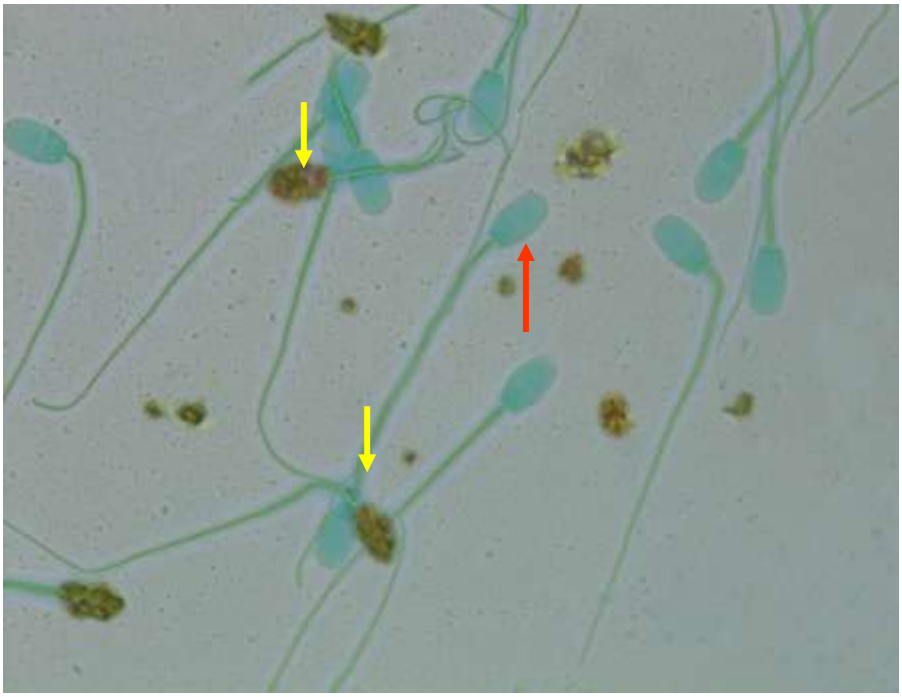

Figure 4. . Apoptotic expression of sperm frozen semen *post thawing* . (1000X)
• spermatozoa undergoing apoptosis appears the brownish color of the sperm nucleus and the cell area prone to shrinkage (yellow arrows)
• spermatozoa do not undergo apoptosis (red arrows)

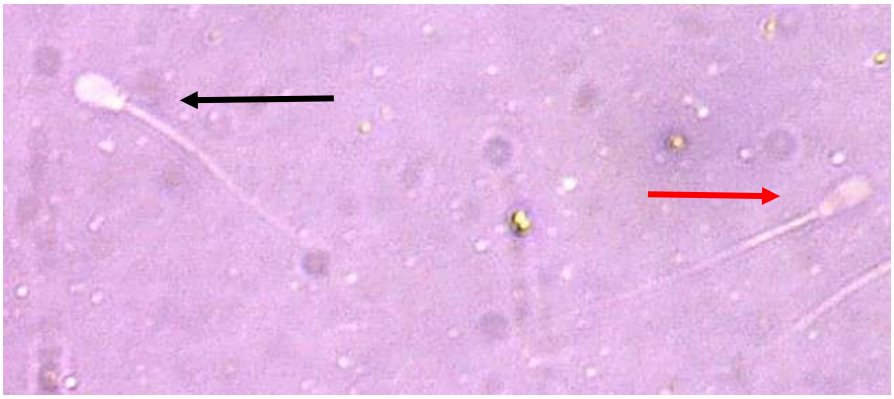

Figure 5. Sperm viability of frozen semen *post thawing* (400X).
• live spermatozoa appear not stained (black arrows)
• dead spermatozoa appear blue-purple (red arrows)

DISCUSSION

in the first stage of the research ,Identification of seminal plasma proteins using SDS-PAGE technique. The results of identification by SDS-PAGE showed bands of protein with molecular weight (MW) is obtained by converting the price Retardation Factor (Rf) is known in the linear regression equation y = 2.313 + -1,284x. for sample 1 to sample 6. The results of this study showed protein bands with molecular weight of 56 kDa were present in all samples. Protein with a molecular weight of 56 kDa which appeared in samples 1 to 6 is osteopontin. SDS-PAGE results of the examination of seminal plasma proteins FH dairy cows is still not specific, so the identification of the protein followed by Western blot techniques to ensure that the 56 kDa protein by weight is an osteopontin. Identification through Western blot technique was known that the protein osteopontin.

The present study no relationship indirectly the effect of osteopontin on post-thawing sperm quality through the mechanism of the studied variables. Osteopontin positive effect on HSP 70, HSP 70 one unit in real viability. Increased viability of the unit followed by an increase in sperm motility significantly . This suggests that administration of osteopontin can increase HSP 70 were followed by a decrease apoptosis, then be followed by an increase in the viability and motility of spermatozoa post-thawing. Freezing sperm was one of the most important techniques in the process of making frozen semen. However, during the freezing temperature and osmolarity changes were extreme. In general, semen freezing problem revolves around the two main problems, namely the cold-shock and intracellular changes due to discharge, associated with the formation of ice crystals This can be explained that the condition occurs when the cell physiological stress such as hyperthermia, hypothermia, hypoxia, hyperoxia, viral infection, the condition of acidosis, Reactive Oxygen Species (ROS), loss of energy, ischemia-reperfusion, activates the inductive form of HSP 70 and increase expression in the cell, (Kregel, 2002). osteopontin may increase the expression of HSP 70 through the interaction between them in maintaining cell resistance to stress caused by freezing spermatozoa. Sequence Arg-Gly-Asp on osteopontin interacts with cell surface receptors, such as integrins. Osteopontin was known to perform adhesion between cells, increasing communication between the extracellular matrix, decrease the production of ROS and nitric oxide in tissue injury experience, as well as alter intracellular calcium levels (Johnson et al., 2003).

Osteopontin especially αvβ3 integrin heterodimer binds to the amino acid sequence of the network through-Glycine-Aspartic Argine Acid (RGD) to hold a bond of communication

between cells and the extracellular matrix. Through its binding to the integrin receptor αvβ3, osteopontin was able to affect cell proliferation, cytoskeletal system settings, motility, apoptosis and phagocytosis in a variety of cells. Osteopontin associated with the fertility of dairy cows FH, with higher numbers in the fluid glands accessories bulls (Killian et al., 1993; Moura et al., 2006). osteopontin work through the membrane surface molecules CD44 and integrin (Weber et al., 1996)

The ability of osteopontin to inhibit the cell death was first identified by Dendahrt et al. (1995), it was known that osteopontin was able to increase the resistance of cells through the barriers against apoptotic cell death (Scatena et al., 1998). The bond between osteopontin with integrin receptors results in translocation of NF-κB to the nucleus that affect the expression of various genes that encode proteins of pro-apoptotic and anti-apoptotic (Saile et al., 2001). NF-κB was a transcription factor that was activated by several cytokines (one of which was osteopontin), then a decrease in the expression of pro-apoptotic factors that increase the resistance of cells to apoptosis.

Osteopontin was an extracellular matrix glycoprotein that was secreted into the seminal plasma (Moura et al., 2006; Moura et al., 2007). Structure of the glycoprotein osteopontin associated with effects on sperm membrane stabilization. It was also related to the interaction with the fat in the form of lipoproteins, causing a more flexible membrane is not easily fragile. Ties between osteopontin, glucose and fat can cause particles accumulate between the membrane, hence a density of the membrane so that the more stable components in the cooling process, freezing and thawing after freezing, the more stable the sperm membrane metabolism running normally, the better the function of spermatozoa.

CONCLUSION

Addition of osteopontin may improve semen quality through increased HSP70 resulting in a decrease in viability and motility of apoptosis that can be maintained.

REFERENCES

Andrabi SMH. 2009. Factors Affecting The Quality of Cryopreserved Buffalo (Bubalus bubalis) Bull Spermatozoa. Reprod Dom Anim 44.552-509.

Anzar M, L Hei, MM Buhr, TG Kroetsch and KP Pauls. 2002. Sperm Apoptosis in Fresh and Cropreserved Bull Semen Detected by Flow Cytometry and Its Relationship with Fertility. Biol of Reprod .66. 354-360.

Aulanni'am,2005. *Protein dan Analisisnya*. Cetakan pertama. Penerbit Citra Mentari Group. Malang. Hal. 19-27

Bilodeau JF, S Blanchette, C Gagnon and MA Sirad. 2000. Levels Antioxidant Defences are Decreased in Bovine Spermatozoa After a Cycle of Freezing and Thawing. Mol. Reprod. Develop. 55 : 282-288.

Brown DG, XM Sun and GM Cohen. 1993. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. Journal Chemical of Biology. 268: 3037-3039

Cancel AM. 1999. Osteopontin Localization in the Holstein Bull Reproductive Tract. Biology of Reproduction 60:454–460.

Chatterjee S, ER Smith, K Hanada, Steven, VL and S Mayor. 2001. GPI anchoring leads to spingolipid-dependent retention of endocyted protein in recycling endosomal compartement. Embo Journal of Medicine. 20:1583-1592

Denhardt DT. 2004. The third international conference on osteopontin and related proteins, San Antonio, Texas, Calcif. Tiss. Int. 74: 213–219.

Hendri. S. 2010. Evaluasi Keberhasilan Inseminasi Buatan pada Sapi Perah Berdasarkan Service Per Conception Tahun 2005 -2009 (Studi Kasus di Wilayah Kerja Koperasi Peternakan Sapi Perah (KPSP) Setia Kawan Nongko Jajar .JawaTimur). Thesis. Universitas Muhammadiyah Malang.

Jeong Y, K Mi, J Hye, J Eun, A Sun, K Mohana, S Balasubramanian and J Gyu. 2009. Effect of α Tocopherol Supplementation on Sperm Characteristics and Expression of Apoptosis Related Genes. Cryobiology.Vol 58: 181-189.

Kacimi R, J Chentoufi, N. Honb, CS Long and JS Karliner. 2000. Hipoxia Differentially Regulates Stress Proteinsin Cultured Cardiomyocytes, Cardiovasc Res;46 (1):139-50.

Kaeoket K, K Tantiparinyakul, W Kladkaew, P Chanapiwat and M Techakumphu. 2008. Effect of Different Antioxidant on Quality of Cryopreserved Boar Semen in Different Breeds Thai Journal of Agricultural Science 41(1-2) : 1-9.

Kamaruddin M, T Kroetsch, PK Basrur, PJ Hansen and WA King. 2004. Immunolocalization of heat shock protein 70 in bovine spermatozoa. Andrologia 36:327–334.

Kankofer M, G Kolm and J Aurich. 2005. Activity of glutation peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5^{o}C. Theriogenology.63: 1354-1365

Kasai M. 1996. Simple and Efficient Methods for Vitrification of Mammalian Embryos. Animal Reproduction Sciences.

Killian GJ, DA Chapman and LA Rogowski. 1993. Fertility-Associated Proteins in Holstein Bull Seminal Plasma. Biology of Reproduction 49:1202-1207.

Kim S and T Shin. 2007. Immunohistochemical study of osteopontin in boar testis. J. Vet. Sci. 8: 107–110.

Kregel KC. 2002. Highligted Topics. Molekuler Biology of Thermoregulation. Inveted Review : Heat Shock Proteins : Modifying Factors in Physiological Stress Responses and Acquired Thermotolerance. J.Appl Physiol 92 : 2177-2186.

Kusumaningrum DA, P Situmorang, E Triwulanningsih dan RG Sianturi. 2007. Penambahan Plasma Semen Sapi dan Antioksidan Gluthatione untuk Meningkatkan Kualitas Semen Beku Kerbau Lumpur (Bubalus bubalis). Seminar Nasi Teknologi Peternakan.

Lenzi A, L Gandini, F Lomabardo, M Picardi, V Maresca and E Panfili. 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione-dependent enzyme-PHGPx from basic to clinic, Contraception. 65:301-304.

Madyawati. 2007. Suplementasi Tyrosin Kinase Spermatozoa Sapi Perah Untuk Meningkatkan Kualitas Semen Beku. Desertasi. Universitas Airlangga.

Martin G, O Sabido, P Durand and R Levy. 2004. Cryopreservation Induces an Apoptosis-Like Mechanism in Bull Sperm. Biology of Reproduction 71:28–37.

Martin G, N Cagnon, O Sabido, G Grizard, P Durand and R Levy. 2006. Kinetics of Occurrence of Some Features of Apoptosis During The Cryopreservation of Bovine Spermatozoa.

Moura, A.A.,H. Koc, D.A. Chapman and G.J. Killian. 2006. Identification of accessory sex gland fluid proteins as related to fertility indexes of dairy bulls: a proteomic approach. J. Androl. 27: 201–211.

Partodihardjo S. 1992. *Ilmu Reproduksi Hewan*. Cetakan ketiga. Mutiara Sumber Widya. Jakarta. Hal. 522 – 556.

Rodriguesz CM, RD Jonathan and GJ Killian. 2000. Osteopontin Gene Expression in The Holstein Bull Reproductive Tract. Department of Dairy and Animal Sci and The Department of Biology. University park. Pennsylvania. 414-419.

Scatena M, M Almeida, ML Chaisson, N Fausto, RF Nicossia and CM Giachelli. 1998. NFκB Mediates $\alpha_v\beta_3$Integrin-Induced Endothelial Cell Survival. J. Cell Biol 1998; 141-1083-93.

Steel RGD and Torie. 1995. Prinsip dan Prosedur Statistika.Penerbit P.T. Gramedia Pustaka Utama Jakarta. Hal. 168-181.

Sudiana. 2008. Patobiologi Molekuler Kanker. Penerbit Salemba Medika.

Suprayogi T, S Abdul dan S Trilas. 2010. Perbaikan Mutu Semen Beku Sapi dengan Penambahan Fertility Associated Antigen (FAA) dalam Media Pembekuan. Laporan Penelitian. Universitas Airlangga.

Sutovsky P, CS Navara and G Schatten. 1996. Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilization. Biol Reprod 55:1195–205

Susilawati T. 2000. Analisis Membran Spermatozoa Sapi Hasil Filtrasi Sephadex dan Sentrifugasi Gradien Densitas Percoll pada Proses Seleksi Jenis Kelamin. Disertasi Pascasarjana Universitas Airlangga.

Tuty LY. 2004. Pengembangan Laboratorium Inseminasi Buatan Melalui Semen Cair dan Beku. Pelatihan Laboran, Puspitnak, Ditjenak, Lembang.