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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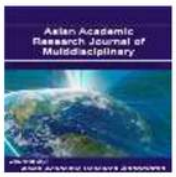

SUPPLEMENTATION OF SPECIFIC PROTEINS IN THE SEMINAL PLASMA FROZEN SEMEN DILUTER MEDIAON THE PLASMA MEMBRANE INTACT, THE EXPRESSION OF CASPASE AND MALONDIALDEHYDE POST THAWING

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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 freezingcementuntilthawing, problems oftenarisecausingcold-shock (cold shock) due to changes intemperature. Cold-shock isone of the causesof oxidative stressdue to increasedReactive Oxygen Species(ROS) excess. thus stimulatinglipidperoxidation(Lenzietal., 2002 andKankofteretal., 2005). AccordingBilodeauetal.(2001), Chatterjeeetal.(2001) andLenzietal(2002), mammalianspermatozoaare rich inunsaturated fattyacidsbindtodoublesoputativelyhighlysensitive toROSThe 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 ended in apoptotic cell death (Green and Reed, 1998).

AccordingSudjana(2008) ROSexcessmust beeliminatedso as not toleadtocell damage. The mechanismfor eliminatingcellsthrough aROSscavengerenzyme (anti-ROS) or so-calledantioxidants, such as superoxidedismutase(SOD) andcatalaseSuperoxidedismutase roletotransformsuperoxide radicalsthat are dangerous tothe cellsto hydrogenperoxide, whilecatalaserole is toconvert hydrogenperoxide to water(H2O) andoxygen(O2). If thescavengerenzymeimpairedtheROSwillbegreaterthan theanti-ROS, thus causingdamageand behavioral changeself. ReactiveOxygen Species(ROS) canbreak thedouble bondsof unsaturated fatty acidsthat make upthe systemacrosomemembraneofsperm cells, forminga substance calledmalondialdehyde(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 acidisa compoundofglutathionewhich is aprimary antioxidant.Wijaya(1996) quoted Triwulanningsihet al. (2003) stated thatglutathioneis atripeptidecontainsthreeaminoacidsthatglutamic acid, glycineandcysteineareprimary antioxidantswhichwork bypreventing the formation ofnew free radicals .Results of research 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 semenfreezing 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 theexpression of caspase and MDA in the post-thawing spermatozoa dairy osteopontin FH after theaddition 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 ofvarious doses of osteopontin treatment technique used immunocytochemistry withmonoclonal antibody caspase. Furthermore colored using immunocytochemistry techniques.Examination of the amount of caspaseexpression in dairy cows performed spermatozoa afterfreezing (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 inPBS 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 addedprimary 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 addedSA-HRP (Horseradish Peroxidase Strepavidin-) for 30-60 min at room temperature after itwas washed in PBS pH 7.4 for 3 x 5 minutes, then added 3,3-diaminobenzidinetetrahydrochloride chromogen (DAB) for 10-20 minutes at room temperature then washed indistilled water for 3 x 5 minutes. Do counterstain with hematoxylin for 5 minutes at roomtemperature, then washed with distilled water for 3 x 5 minutes. Further mounting withentellan. Observation using an optical microscope with 400x magnification (Nurhidayat,2002).

Measurement of levels MDA(Malondialdehyde) PostthawingthespermDairy CattleFHafteraddition ofOsteopontininSemenFreezingMedia

A total of 100mL ofspermsuspensionis separatedfromthe proteinby the addition of100mL of20% trichloroacetic acidandvortexfor 30seconds, thenadd250mL of1NHCl, 100mL of1% thiobarbituricsodiumanddistilled water untilthe final volumeto 1ml(450µl) Then heatedin awater bathat a 100^{o}Cfor 20 minutesandcentrifugationat a speed of3500rpmfor 10 minutes. Seanyak800mL ofthesupernatantis insertedinanother tubeand adddistilled watertoa final volume of2ml. Uptakecolorread usinga spectrophotometerat a wavelength of529nm. Rateof MDAis doneby convertingthe value ofthe absorbancemeasurement resultswith thestandard curvevaluestandarpureMDAindifferentconcentrations Furthermore,the result of multiplyingthevalue ofthe gold standardcurve, multipliedby the dilution factorused. MDA levelsmeasured bytheMDAnmolgorpermlsuspension ofspermatozoaorspermatozoaconcentrationperml

MembraneIntegrityExaminationPresentationPostthawingspermatozoaDairy CattleInFHafteraddition of Osteopontin in Semen Freezing Mediavia Hipoosmotic Swelling Test Method (HOS Test)

Examination ofcell membrane integrityof spermatozoapresentationwas conducted byhypoosmoticswellingtest (HOST) developed by Jayendraetal. (1984). Suspensionspermatozoafrozen semenoriginatingfromcowsthat have beenadded tovariousconcentrations ofosteopontin(T0, T1, T2, T3, T4) is takenas0.1mland add9.9mlof 0.032Mhypoosmotik(prepared from 7.35gof sodiumcitrate2H2O, 13.52gfructosedissolved in1literof distilled water).Furthermoreincubated for1hourin aCO2incubatorat 37 ° C.Thena thincovermadepreparationsby mixinga drop ofthe abovesolutionwithone drop ofeosinandobserved with alight microscope400x magnification. Spermatozoahaveplasmamembrane integrityintacttail sectionvisiblepresence ofswellingfollowedthe tailrotateswith a radiantlight colors, while spermatozoawithdamagedplasmamembraneis characterizedby the absence ofswelling ofthe headwitha straight tail.

RESULT

Osteopontintreatmentof theMDAshow, P0significantly different fromT1($p>0.05$), andhighly significanttoT2, T3andT4($p <0.01$). The same thing wasseen inT1toT2, T3andT4whichshows the difference inMDAwere significantly ($p <0.01$).OsteopontintreatmentatT2toT3andT4showed significant differencesagainst($p <0.01$),. T3toT4osteopontintreatmentshowed nosignificantdifference inMDA($p>0.05$).Osteopontintreatmentagainstcaspaseshows thatT0toT1was not significantly different($p>0.05$) butsignificantly differentwithT2, T3andT4) $p<0.01$). T1is notsignificantly different fromT2andT3($p>0.05$),butsignificantly different withT4($p <0.05$). T2is notsignificantly different from theT3andT4($p>0.05$). The same thingwas alsoshown byT3andT4($p>0.05$). 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 onosteopontintreatmentT1highly significantboth toT2, T3andT4($p <0.01$). T2toT3orT4did notshow significant differences($p>0.05$). Similarly, betweenT3andT4showed no differencePMIsignificantly ($p>0.05$). The result ofthe calculation ofosteopontintreatmentof thePMI can be seenin Table1The results ofthe examinationPMIspermatozoapresented in the figure2

Table 1.Osteopontin treatment on level MDA ,expressioncaspase 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]

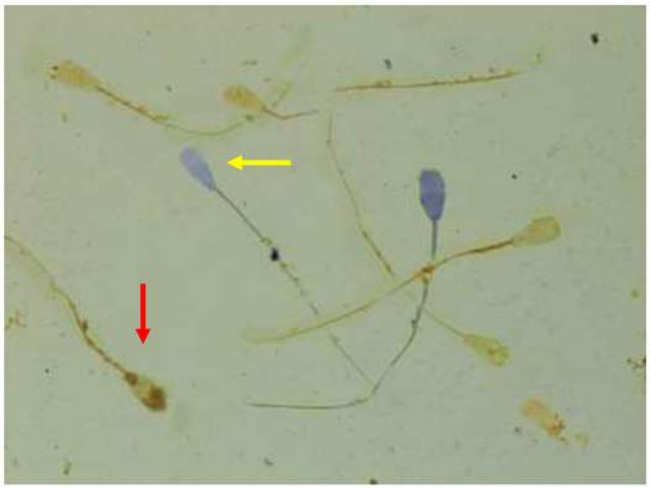

Figure 1. .*caspase*-9 expression of sperm frozen *postthawing*.(1000X)
(A) spermatozoaexpressingcaspase-9 appears thebrownish color, especially in thecore area ofspermatozoa(red arrows)
(B) spermatozoado not expresscaspase-9 (yellow arrow).

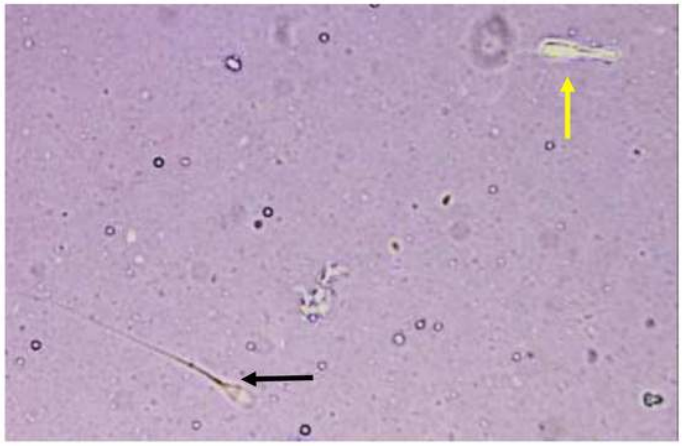

Figure 2.PMIexamination resultsspermpostthawingof frozen semen.
A)damaged sperm plasmamembrane(black arrow)
B) sperm withintact plasma membrane(yellow arrow)

Discussion

Effect ofosteopontinon levels ofMDAspermatozoaafterthawingfrozendairy cowsFreezingspermis one of themost importanttechniquesin the manufacturing processof frozen semen. However, duringthe cryopreservationtemperature changeandextremeosmolality.In general, semenfreezing problemrevolves around thetwo main issues,namelycold-shock andintracellularchangesdue todischarge, which is associated with the formation ofice crystals(Mumu, 2009).

The results ofthis study indicate thatamongthe group withoutaddition ofosteopontinsignificantly different from thegroup of5ugosteopontinadministration, andhighly significantto thegroup ofosteopontinadministration of10ug, 15ugand20ug. The same thing wasseenin the group ofosteopontinadministration of5ugto10ug, 15ugand20ugof demonstratingthe very realdifferences inMDA.Group ofosteopontinadministration of10ugagainst theadministration of15ugand20ugshowed significant differences, osteopontinadministration of 15uggroupagainstosteopontinadministration of20uggroupsshowed nosignificant differencein decreasingMDA.

This proves that the addition of osteopontin with a dose of 15 ug 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) MechanismspermatozoabyROSdamageis induced lipid membranein spermatozoaandreactif the formation oflipidperoxidation(LPO). Susceptibility tocold temperaturesassociatedwiththe ratio ofunsaturatedacid contentis high (Poly-unsaturated Fatty Acid/PUFA) than saturated fatscontained inthe spermplasmamembrane,Cholesterolis one ofunsaturated fatty acidsthat make up thespermplasmamembrane. Osteopontinaddedhavingthe aminoacid sequencecontainsone of whichserves to maintainthe conditionredokofoxidation of proteinsthroughthereversibleoxidationofthe amino acidNordbergandArner(2001). TerminalSequenceAnalysisofosteopontinproducesa sequence of15aminoacids(VKPXSSGXSEEKQLN) with85% homologoustoknowncattledeoxyribonuclease(DNase) I-like protein. (Cancel, etal., 1997). AccordingFeradis(2009) is themost important component ofthe cell membranephospholipids, glycolipidsandcholesterol. The first two componentscontainingpolyunsaturated fattyacidsthatare highly susceptible tofree radical attack, especiallyhidrosilradical(OH *). Thesehydroxyl radicalscancause a chain reactionknown aslipidperoxidation. According toBucak etal(2010) sperm cellscontain ahigh content ofpolyunsaturatedfattyacids, makingthe membranemore susceptible to damageperoxidation. Spermatozoamembrane is expected to be

themain targetorcoldshockfreezing damageto cells atthe processing offrozen semen,cryopreservationhas beenknown togenerateReactive OxygenSpecies(ROS). ROSis one ofoxidantsorfree radicals, in which themain properties of thefree radicalitselfis to have oneormoreunpaired electronsintheir outermosttrack. This has resulted inROSinto components that arereactivewithcertainsubstances. Atnormallevelsin the sperm, ROSare veryhelpfulin the process ofhyperactivation, capacitation, acrosome reaction, andbindingofthezone(zone bindings) (Kodama etal, 1996; deLamirandeetal, 1997).

Some studies show thatROSplay animportant role infertility/infertility insperm. When thebalance betweenROSproductionanddetoxificationby antioxidantsinterrupted, excessROStriggeroxidative stress.ROS(one of which isH2O2) is knownto function asholdmotilityandspermblockoxidative 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 decoxahexanoid acid (DHA) membranes, therefore the increase in MDA in suspension is generally used as one indicators for membrane lipid peroxidation (Alvarez and Strorey, 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 disturbed.SequenceArg-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).AccordingBaldietal(1996) andAmin(2000) to highintracellular calcium concentrationwillincrease the activity ofthe enzymeadenylatecyclase,thereby increasingcAMP concentrationensuingphosphorylationenzymesleading to highmotility.OsteopontinaswithvitaminEas an antioxidantcanreduce levels ofMDA(Agarwal.A.2005). Osteopontincontaininggammaglutamylacidcan act as ascavengerthat inhibitslipid peroxidationreactionso that themembranescanlowerthe levels

ofMDA. A similar mechanismis alsofound inthe activityof glutathioneinhibitmembranelipidperoxidation(Barycki. 2008)

Effect ofosteopontinon the expression ofcaspasespermatozoaafterthawingfrozendairy cows.The results showed thatwithoutthe addition ofosteopontinagainstcaspase9was notsignificantly different from thegroup ofosteopontinadministration 5 ugbuthighly significantto thegroupgiving10ug, 15ugand20ug. 5ugosteopontinadministrationgroupwas notsignificantly different from thegroup ofosteopontinadministration of10ug and15ug, butsignificantly different from thegroup ofosteopontinadministration of20ug. Osteopontinadministration of 10uggroupwas notsignificantly different from thegroup ofosteopontinadministration of of20uggroup The same thingis also shownby the group ofosteopontinadministration of15ugand20ugosteopontinadministrationgroupswere not significantly different. This result meansa dose of10ugosteopontinadministrationaloneget theexpression ofcaspase9reduced the percentageofspermatozoa.

The results ofthisresearchwassupportedbytheresults of research conductedbyMartinetal. (2006) who observed thattheprocessof cryopreservationorthawingcauses a decrease viabilityasassociatedwith the incidence ofapotosis. It isbased onthe change inthe mitochondrial membrane, causing an increase incaspase-9 andBax, Bcl-2butfactorinthe studies were notdetected.

Thisstudywas not detectedoncaspase3, in linewith Martinetal. (2006), the expression ofcaspase-3 was not detectedin the study.In addition, studies have beencarried outbyHendricksandHansen(2009) observed inthe apoptoticcell death pathwayspostejaculatedspermatozoain thetwospecies, namelyhorses and cows.Boththecow andhorsespermatozoa, proactivecaspase-9 that caspase3is detected butnot detected. Through theintegrinreceptor, osteopontinable to activateNF-κBisassociated with the expressionofvariousgenes that encodeproteinspro-apoptotic andanti-apoptotic (Saileetal.,2001).

Effect ofosteopontinon theplasmamembraneintact(PMI) of the spermpostthawingfrozendairy cows .Boththe plasma membraneandmitochondrial membrane of sperm cattlesusceptible to the influenceof cryopreservation(O'Connell etal., 2002). The maininfluenceon thecryopreservation ofsperm cellsisa decrease inmotilityandvitality, permeabilitychangesand changesin the membranelipidcomponents The maininfluenceon thecryopreservation ofsperm cellsisa decrease inmotilityandvitality, permeabilitychangesand changesin the membranelipidcomponents. The onset oflipid peroxidationduringsemenfreezing processaffectsthe cellmembrane damageinspermatozoa.

Physical damageone of whichcan be eitherplasmadamageandmembrane acrosome(Ismaya, 2009) MPUosteopontintreatmenttoshowthat the groupwithoutgivingosteopontinagainstosteopontinadministration of5uggroupdid notshow significant differencesbutwithoutgivingosteopontingroupagainstgroup ofosteopontinadministration of10ug, 15ugand20ugshoweda 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 thegroup ofosteopontinadministration of15mgand20ugosteopontinadministrationgroupsshowednosignificantdifference inapoptosis. The results ofthis studydemonstratethat administration ofa dose of 5ugosteopontinhas not been abletomenstabilitaskanspermmembranefrom aroundspermatozoaandprovethe addition ofosteopontinwitha dose of 15uganoptimaldosein reducingthe percentage ofapoptosisin the process offreezingsperm

The plasma membraneis thecell wallmembranewhich controls theexit and entry ofsome substancesthat are required inthe process of metabolismandactivity oflivingcells.The plasma membraneis composedofprotein, carbohydratesandfatsthat can actas a receptorfor aparticular compound. Plasmamembraneintactspermatozoawhichserves as a protectiveoverall cellsurvivalspermatozoa.In addition, the integrity of thespermmembranealso actsas a protectorof cellular organellesofmechanicalor chemicaldamage, filterfor the exchangeintra-andextracellularcompoundsareretainedin the metabolic processes(Garner andHafez, 2000). Membraneintegrity ofspermatozoais also appliedin the evaluation ofthe quality ofspermatozoain the cement industryof frozen(Kennedy andSutovsky, 2011)

The membraneconsists ofbiomolecularlipidlayerwithproteinsinsertedin itorattached to onesurface of themembrane. Integralmembrane proteinsembeddedinthe lipid layerandstrong. most ofthese proteinsare fullystretchedanddouble layercalledtransmembrane proteins, while othersare embeddedinthe outer layerordouble layerof lipids. Peripheralproteinsboundlooselyonthe internalsurface ofthe membrane. many oftheproteinsandlipidsthathaveoligosaccharidechainexposedout(Murray andGanner, 2001).

Osteopontinis anextracellularmatrixglycoproteinthat issecreted into theseminalplasmaandliquidaccessoryglands(Mouraetal., 2006; Mouraetal, 2007). The composition ofosteopontinglycoproteinassociatedwith the effect onspermmembrane stabilization. It isalsorelated to the interactionwith the fatin theformof lipoproteins, causing the membranesmore flexibleis not easilyfragile. Tiesbetweenosteopontin, glucoseand

fatcancause the particlesbetweenthe membraneis collected,hence adensityofmembrane componentsso that morestablein the process ofcooling, freezingandthawingagain afterfreezing, the stability ofthe membraneof spermatozoa, the metabolismis running normally, the function ofspermatozoaintobetter. The results are consistentwithresearch conductedbySuprayogi(2013)which usesFAAcontainingglycoproteins. ,Showedthat the addition ofosteopontineffectivein protectingspermatozoa inspermfreezinguntil it isthawing

CONCLUSION

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

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