In vitro fresh sperm preparation for maintaining sperm viability at storage temperature of 14°C using tannin supplementation of lamtoro leaves

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ABSTRACT: A research on in vitro fresh sperm preparation for maintaining sperm viability at storage temperature of 14°C using tannin supplementation of lamtoro leaves (*Leucaena leucocephala*) had been carried out in both Physiology and Animal Reproduction Laboratory, Faculty of Animal Science, Universitas Gadjah Mada for fresh sperm preparation and sperm quality evaluation, during March to July of 2009.The experiment was designed using the Complete Random Design consisted of 5 treatments and 5 replications. Variables measured were viability, motility and morphology of the treated fresh sperm stored for 14 days at temperature of 14°C. Based on the analysis of variance, the treatments had significant effect ($P<0.05$) on viability, motility and morphology of the sperm. In terms of viability, P1(64.07%±0.45) it was higher than control (P0=26.71%±0.54), but it was not different for P2(57.44%±0.52), P3(49.49%±0.71), and P4 (47.83%±0.61). The progress and very progress motility of spermatozoa were only found in control, and not in others. Non-progressive spermatozoa proportion of P1, P2, P3 and P4,was found respectively. They were significantly higher than control.For the morphology, P4 had lower normal spermatozoa percentage than P0, P1, P2 and P3, whereas among P0, P1, P2, and P3, there were not different ($P>0,05$).

Key words: fresh sperm preparation, sperm quality, viability, tannin, Lamtoro leaves

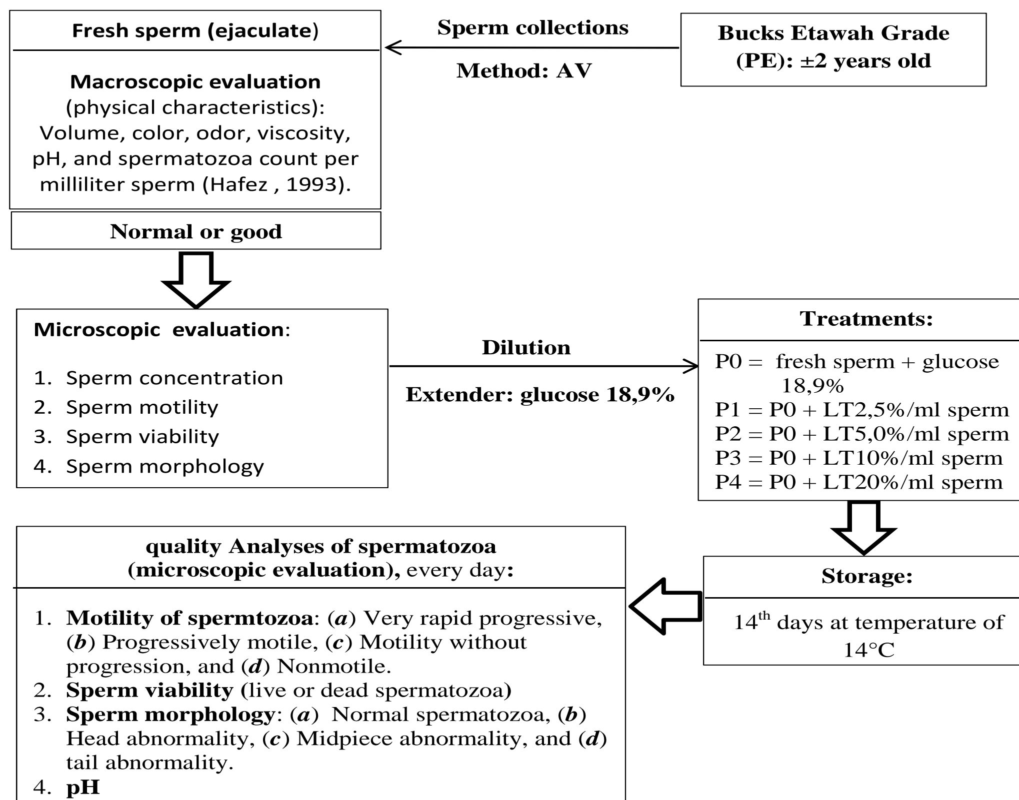
INTRODUCTION

Behaving aim changing and global livestock developing, it is necessary to develop strategy reorientation of livestock and to provide national flesh. Thus, government ought to give greater emphasis on livestock production process; notably developmental and increasing local breeds productivities that exist at region sort at Indonesian as breed of potential domestics and at a swoop keep their existence as nutfah's plasma. The Central Sulawesi Province, has local breeds with tall genetics potency for adaption on production environment in point appropriate its development region, so it constitutes main judgment in breeding production system intensifications domestic sustainables.Developments classic problems and livestock development at this region as well as at Central Sulawesi Province, still addressed on its low local breeding productivity, and its population on the wane. In the meantime, Artificial insemination (AI) constitutes solution for expedited productivity and its population, but then on its performing, it is 'fail'. Thus, necessary strategics steps to perform AI's technology with by extenfication, effective and continually. AI's performing the failing as one of Central Sulawesi, more because of the existence should be factor and frozen sperm limitation and liquid-Nitrogen (N_2), causing alow number of AI's yielding gestation (<5%).The sperm preservation methods, of both frozen and fresh sperm showed a low conception rate when the sperm was artificial inseminated to the does. It was proposed that it had relation to the lower viability and fertility of the sperm. An alternative method in preparation of fresh sperm is therefore suggested to solve the problem.Fresh sperm when ejaculated do not capacitation, because they contain the decapacitation factors. Accordingly, to keep the fresh sperm not iscapacitation it is one way to preserve the fresh sperm. It is hypothesized that supplementation leucocephala tannin can constrain capacitation spermatozoa by ties-up and keep decapacitation factors on seminal and spermatozoa membrane. The objective of this study was to examine at which level of the supplementation of leucocephala tannin was able to keep decapacitation in order that the fresh sperm was preserved at 14°C.

MATERIALS AND METHODS

***Materials*.** **(1)** Fresh sperm (fresh ejaculate), gotten of three bucks of EttawaGrade (±2 years old). Sperm collection, was done by artificial vagina method. (2) Tannin lamtoro's leaf/Tanninleucocephala (TL),gotten by extraction of lamtoro's leaf (dry sample) with butanol HCL's method (FAO / IAEA, 2000). (3) Glucose solution 18,9%, as extender. (4) NaCl 3% and 9%, alcohol 70%, Eosin, and aquadestilata.

***Method*.** The research method was the following diagram:



Experimental design. The experiment was designed using the Completely Randomized Design, consisted of 5 treatments and 5 replications. The treatments tested were as follow:

1. P0 = fresh sperm + glucose 18,9% + LT0,0%, as control.
2. P1 = fresh sperm + glucose 18,9% + LT2,5%,as 1st treatment.
3. P2 = fresh sperm + glucose 18,9% + LT5,0%,as 2nd treatment.
4. P3 = fresh sperm + glucose 18,9% + LT10%, as 3rd treatment.
5. P4 = fresh sperm + glucose 18,9% + LT20%, as 4th treatment

Parameters

Normal values of ejaculate variables.

Sperm that was assessed by normal only that given by conduct.Evaluation of ejaculate, with initial macroscopic examination,was as follows:

a) Volume, measured after sperm collection (method: artificial vagina) which was reads on collection tubes scale.
b) Color
c) Odor
d) Viscosity, its estimation was done by seeing wave or spermatozoa's term movement upon a glass object
e) pH, measured by using of pH meter, and
f) Spermatozoa count per milliliter.

Quality of Spermatozoa

Concentration. Sperm concentrationrefers to the number of spermatozoa per milliliter of semen. The sperm count is the total number of spermatozoa in the ejaculate. Both are important and should be calculated.The concentration of spermatozoa should be determined using the haemocytometer method. In this procedure a 1:20 dilution is made from each well-mixed sample.

Motility.Motility of spermatozoa is utilized as measure of spermatozoa's capacity to fertilize the egg.The microscopic field is scanned systematically and the motility of each spermatozoon encountered is graded 'a', 'b', 'c', or 'd', according to whether it shows:

'a' : rapid progressive motility
'b' : slow or sluggish progressive motility
'c' : nonprogressive motility
'd' : immotility.

***Morphology*.**Morphology constitutesthe measure of a spermatozoa's form, normal or abnormal. The microscopic field is scanned systematically and the morphology of each spermatozoon encountered is classified as 'a', 'b', 'c', or 'd', according to whether it shows:

'a': Normal spermatozoa
'b' :Head abnormality (head shape/size defects, including large, small, tapering, amophous, vacuolated, or double heads, or any combination of these. WHO,1992).
'c' :Neck and midpiece defects, including absen tail (seen as free or loose heads), non-inserted or 'bent' tail (the tail forms an angle of about 90° the long axis of the head), distanced/irregular/bent midpiece, abnormally thin midpiece, or any combination of these (WHO, 1992).
'd' : Tail defects, including short, multiple, hairpin, broken (angulation >90°), irregular width, or coiled tails or tails with terminal droplets, or any combination of these (WHO, 1992).

The number of spermatozoa in each category can be counted with the aid of a laboratory counter. Usually four to six fields have to be scanned to classify 100 successive spermatozoa, yielding a percentage for each motility and morphology category. The count of 100 spermatozoa is repeated and the average values calculated for each category. The values are expressed as percentages, adding up to 100 (WHO, 1992).

***Viability* (*sperm vitality*).** Sperm vitality is reflected in the proportion of spermatozoa that are 'alive' as determined by either dye exclusion or osmoregulatory capacity under hypo-osmotic conditions (WHO,1992).

Sperm vitality staining techniques: Eosin alone (WHO,1992).
Procedure:

1. Mix one drop (10-15 µl) of fresh sperm with one drop of 0,5% (5 g/l) eosin solution on a microscope slide and cover with a coverslip.

2. After one or two minutes, observe the preparation at 400X under bright light or phase contrast.
3. Count unstained (live) and stained (dead) spermatozoa as described in the text.

One hundred spermatozoaare counted under the light or phase-contrast microscope, differentiating the live (unstained) spermatozoa from the dead (stained) cells. These staining techniques make it possible to differentiate spermatozoa that immotile but alive from those that are dead.

These tchniques also provide a check on the accuracy of the motility evaluation, since the percentage of dead cells should not exceed the percentage of immotile spermatozoa. Furthermore, the presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum (WHO, 1992).

Statistical analysis

Spermatozoa's quality data as analysed by the use of analysis of variance (Anova) of one way. The significant different (P<0,05) or the highth significant different (p<0,01), was treated by Polynomial Contrast Ortogonal. Normal values of ejaculate variables data before being given by conduct, were analysed by descriptive analysis.

RESULTS AND DISCUSSION

Fresh Sperm (Fresh Ejaculate)

Fresh sperm datafrom macroscopics evaluation,were shown in Table 1. The fresh sperm sample was then evaluated by microscopic's method, diluted (liquefaction) by glucose solution of 18,9% to increase sperm volume according to the requirement. Microscopic's evaluation result of average spermatozoa's quality parameter of fresh sperm was the***concentration*** of ±328,8X10^7 /ml sperm, **motility** (category (%): ***a***= 19.4***;*** ***b***= 31.0***;*** ***c***= 22.0***;*** ***d***= 26.6), **morphology** (category (%): ***a***= 66.6***;*** ***b***= 6.6***;*** ***c***= 7.8***;*** ***d***= 16.8) and average **spermatozoa lives** are 77%.

Tabel 1. Average Normal Value of ejaculateup to Research

Parameters	Value
Volume	1,4 milliliter
Color	Creamy
Viscosity/consistency	Normal
Odor	specifics
pH	6,84
Appearance	Normal
Spermatozoa count per milliliter	328,8 x10^7/ml

Quality of Spermatozoa. The Spermatozoa's quality data at storage temperature of 14^{0}C for 14 days, were shown at Table 2.

Viability of Spermatozoa. The proportion of live to dead cells can be estimated by supravital staining with a stain mixture such as nigrosin-eosin (Hafez, 1993) or eosin alone (WHO,1992). The cells that were alive when the stain was applied exclude the stain, and the dead cells stain red with eosin against the dark nigrosin background. The results were highly correlated with visual estimates of progressively motile cells, but the latter averages were lower than the percentage of unstained spermatozoa.Fresh sperm preparation *in vitro* with supplementationof tannin lamtoro leaves (leucocephala tannin) by concentration 2,5% - 20% per milliliter sperms, showedhighlyspermatozoa's percentage lives (P<0,05) at 14°C temperature storage up to 14 days against the fresh sperms without supplementation. Where abouts, supplementation leucocephala tannin (LT)2,5% apparently had the ability to keep spermatozoa better lives (P>0,05) and another concentration application.It could be aforted that tannin lamtoro's leaf, can tie-up complex or protein that was bound by Ca ion's, Mg, Na

and K; carbohydrate, and fat (Rusdi, 2003), also constituted as decapacitation factors on seminal and spermatozoal membrane, one that prevented premature of capacitation (Hill, 2008; Anonimous, 2007)and thus, the fertilityof sperm (Kawano and Yoshida, 2008);Physiologically, it was achieved through spermatozoal membrane stabilization by maintaining physiological cholestrol/phopholipid ratio (Anonimous, 2007), so spermatozoal death prematurely had to be prevented.Decapacitation factor is composed of factors in seminal plasma which modulates the fertilizing ability of spermatozoa. The activity is achieved by interaction between cholesterol, phospholipids and fibronectin-like substances and delivered via sall vesicles in seminal plasma(Hill, 2008; Anonimous, 2007). Decapacitation factorcan as glycoprotein, sterol, or may be lipids (Zaneveld, *et al*.,1996).Perfection and membrane stability can also prevent*influx*ion ($Ca^{2+,}$ Na^{+} , and Cl^{-})and *efflux* Ca^{+2} on membrane (Baldi, *et al*., 1996), so it gets to press activity step-up reacts peroksidatif who can wreck and switches off spermatozoa (Anonimous, 2007). On the other hand, diluted fresh sperm with glucose solution of 18,9% single without supplementationof leucocephala tannin, could not keep viability of spermatozoa in the period of relatively so long on 14°C's temperature storage. It was caused, that glucose purpose as extender, functioned as source of spermatozoa's energy (Hafez, 1993), although the seminal gland's secretion contained various proteins and fructose as energy suppliers for sperm motility and was also responsible for making the largest proportion of the alkalinebuffer.The prostate secretion contains protease for fluidizing the ejaculate, which supported the motility and the further maturation of the spermatozoa (Mitchell, *et al*., 2007), but it could not keep spermatozoa's membrane stabilityso could not press activity step-up of peroxidative reactions(Breitbart and Naor, 1999) who beget spermatozoa's damage and death.

Table 2. Spermatozoa's quality Average among conduct on stored 14°C's temperature up to 14 days.

Parameter of Quality	Treatments				
	P0	LT2.5% (P1)	LT5.0% (P2)	LT10% (P3)	LT20% (P4)
Motility of Spermatozoa (%)					
Very rapid progressive	$1.57{\pm}0.31^{a}$	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Progressively motile	$3.66{\pm}0.29^{b}$	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Motile-Non progressive	$15.86{\pm}1.20^{a}$	$47.90{\pm}0.82^{b}$	$41.40{\pm}1.36^{b}$	$41.67{\pm}1.62^{b}$	$31.97{\pm}1.18^{ab}$
Nonmotile	$78.80{\pm}0.70^{a}$	$51.96{\pm}0.80^{ab}$	$59.14{\pm}0.80^{ab}$	$58.01{\pm}1.06^{ab}$	$68.03{\pm}0,76^{ab}$
Morphology of Spermatozoa (%)					
Normal	$84.8{\pm}0.42^{a}$	$83.4{\pm}0.34^{a}$	$79.8{\pm}0.34^{ab}$	$79.4{\pm}0.34^{ab}$	$67.4{\pm}0.61^{b}$
Head Abnormality	$7.6{\pm}1.46^{a}$	$9.4{\pm}1.37^{a}$	$11.2{\pm}0.89^{a}$	$15.4{\pm}0.92^{a}$	$25.6{\pm}1.61^{a}$
Midpiece Abnormality	$5.6{\pm}1.03^{a}$	$5.0{\pm}0.59^{a}$	$4.4{\pm}0.48^{a}$	$2.6{\pm}0.59^{a}$	$4.2{\pm}1.01^{a}$
Tail Abnormality	$2.2{\pm}0.71^{a}$	$2.6{\pm}0.48^{a}$	$4.6{\pm}0.26^{a}$	$2.6{\pm}0,39^{a}$	$2.6{\pm}1.12^{a}$
Precentage live of Spermatozoa	$26.71{\pm}0.54^{a}$	$64.07{\pm}0.45^{b}$	$57.44{\pm}0.52^{b}$	$49.49{\pm}0.71^{b}$	$47.83{\pm}0.61^{b}$
pH	$5.52{\pm}0.80^{a}$	$5.46{\pm}0.23^{a}$	$5.23{\pm}0.26^{a}$	$4.94{\pm}0.34^{a}$	$4.95{\pm}0.26^{a}$

[ab] Spermatozoa's quality point that followed by superskrip is different point out a marked difference (P<0,05) and same superskrip not different reality (P>0,05);
LT: Leucocephala tannin per millilitersperm.

Motility of Spermatozoa. Results indicated that treatments affected the motility of spermatozoa stored at 14°C for 14 d. Rapid progressively (category ‘a’) and progressively (category ‘b’) motile spermatozoa were both found only for the control treatment, while the non-progressive spermatozoa (category ‘c’) was found to be significantly higher for treatments P1, P2, P3, and P4 than for control (P0). This was probably because the leucocephala tannin in P1, P2, P3, and P4 was able to prevent capacitation by maintaining or binding decapacitating factors on spermatozoa plasma membrane. Thus, increased intracellular concentration of Ca^{2+} ion required to stimulate *adenylate cyclase* did not occur as it is needed to support cAMP for changes in physiological characters of plasma membrane

(Parrish and First, 1993) that will enable energy production for the spermatozoa to become rapid progressivelyor progressively motile.

Morphology of Spermatozoa. Percentage of spermatozoa with normal morphology was to be significantly higher ($P<0.05$) for P1, P2, P3 or P4 than for P0 (control). This probably because that spermatozoa in tannin supplemented sperm was able to maintain its membrane stability by maintaining its membran cholesterol/phospholipid ratio (Hill, 2008), considering that condensed tannin is capable of binding proteins which have high affinities, large and open molecules (Rusdi, 2003). Tannin is also able to bind to complex proteins, including those with Ca, Mg, Na, and K ions as well as carbohydrates and lipids in their molecules. However, molecular basis for this phenomenon requires more investigation.

CONCLUSION

In vitro fresh sperm preparation method with supplementation of leucocephala tannin 2,5% per milliliter sperm with extender glucose 18,9%, can keep viability and normal morphology of spermatozoa storedat 14°C for 14 days, the better (64,07±0,23% and 83,40±0,34%), as compared to another conduct; and can be used to replace in the presence frozen sperm for AI at region that have limitation problem frozen sperm and liquid-N2 on AI's performing.

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