Epididymal Sperm Quality of Buffaloes with Different Spotted Types

Yulnawati Y[1,2], Rizal M[3], Maheshwari H[4], Noor RR[5], Sumantri C[5], Boediono A[4]

[1] *Graduate School, Bogor Agricultural University (IPB),*
Jl. Agatis IPB Campus, Darmaga, Bogor, Indonesia
[2] *Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI),*
Jl. Raya Bogor Km. 46, Cibinong, Indonesia;
Email: yulnawati@yahoo.com
[3] *Dept. of Animal Science, Fac. of Agriculture, Lambung Mangkurat University,*
Jl. Jenderal Ahmad Yani Km 36, Banjarbaru, Indonesia
[4]*Dept. of Anatomy, Physiology and Pharmacology, Fac. of Veterinary Medicine, Bogor Agricultural University (IPB),*
Jl. Agatis, Kampus IPB, Darmaga, Bogor, Indonesia
[5] *Dept. of Animal Production and Technology, Fac. of Animal Science, Bogor Agricultural University (IPB),*
Jl. Agatis, Kampus IPB, Darmaga, Bogor, Indonesia

(Diterima 5 Juni 2013 ; disetujui 14 Agustus 2013)

ABSTRAK

Yulnawati Y, Rizal M, Maheshwari H, Noor RR, Sumantri C, Boediono A. 2013. Kualitas sperma kerbau belang dengan tipe berbeda. JITV 18(3): 202-207. DOI: 10.14334/jitv.v18i3.322.

Populasi kerbau belang mengalami penurunan yang sangat signifikan dalam beberapa tahun terakhir ini, sehingga menyebabkan spesies ini terancam mengalami kepunahan. Untuk mencegah hal tersebut, maka studi guna memperoleh data dasar reproduksi dan genetik kerbau belang perlu dilakukan. Tujuan penelitian ini adalah untuk mengetahui kualitas sperma epididimis berdasarkan variasi warna kulit kerbau, sehingga selanjutnya dapat dilakukan kajian lanjutan terhadap gen yang spesifik sebagai kandidat kuat pembawa mutasi yang menyebabkan munculnya warna belang. Dalam studi ini, kami membandingkan kualitas sperma segar dan *post-thawing* dari 12 ekor pejantan belang (yang terdiri dari tiga kelompok tipe belang berbeda, yaitu *Saleko, Bonga, dan Lotong Boko*), dengan sperma dari lima ekor pejantan hitam (normal). Hasil yang diperoleh menunjukkan bahwa tidak ada perbedaan signifikan ($P > 0.05$) pada semua parameter yang diamati, baik pada semen segar maupun *post-thawing*. Persentase motilitas progresif *post-thawing* dari kelompok *Saleko, Bonga, Lotong Boko dan* Hitam, berturut-turut adalah 44%, 42%, 40% dan 42%. Sementara itu, daya tahan hidup dan keutuhan membran plasma *post-thawing* dari keempat kelompok tersebut adalah 64,9%; 65,2%; 62,6%; 62,7% dan 64,6%; 67,1%; 64,5%; 64,1%. Dapat disimpulkan bahwa kualitas sperma epididimis kerbau belang tidak dipengaruhi oleh perbedaan warna kulit.

Kata Kunci: Sperma Epididimis, Kerbau Belang

ABSTRACT

Yulnawati Y, Rizal M, Maheshwari H, Noor RR, Sumantri C, Boediono A. 2013. Epididymal sperm quality of buffelous with Different Spotted Types. JITV 18(3): 202-207. DOI: 10.14334/jitv.v18i3.322.

The significant decline of spotted buffalo population nowadays brought this species into an endangered situation. To perform an integrated conservation project, we need some basic information and data related to the reproductive and genetics potency of this buffalo. The purpose of this study was to observe the effect of coat color variation to the sperm quality, in order to get focus on specific candidate gene that allegedly bring the causative mutation(s) and responsible for the different pigmentation expression. In this study, we compare the quality of fresh and frozen-thawed epididymal sperm from 12 spotted bulls (that classified in 3 different spotted types, *i.e Saleko, Bonga,* and *Lotong Boko*) with five solid bulls. The results showed that there were no significant differences ($P > 0.05$) in all parameters of fresh and frozen-thawed epididymal sperm among those groups. The percentage of frozen-thawed progressive motility from *Saleko, Bonga, Lotong Boko,* and Solid was 44%, 42%, 40% and 42%, respectively. Moreover, the percentage of livability and membrane integrity of frozen-thawed sperm from each groups were 64.9%; 65.2%; 62.6%; 62.7% and 64.6%; 67.1%; 64.5%; 64.1%. In conclusion, it suggested that the coat color/phenotype difference has no effects on the quality of fresh and frozen-thawed epididymal sperm of spotted buffalo.

Key Words: Epididymal Sperm, Spotted Buffalo

INTRODUCTION

Spotted buffalo (*Bubalus bubalis carabanensis*) mostly exists in Tana Toraja, South Sulawesi Province, Indonesia. Their population is very limited and getting closer to extinction. Although it is known that spotted buffalo is also found in a very small number in other region such as Central Sulawesi, Sumba, Flores, Roti and Timor (Bo'do', http://sulawesi.cseas.kyoto-u.ac.jp/final_reports2007/article/212-stephanus.pdf).

The small number of spotted buffalo is caused by the unbalance of delivery rate compare to slaughter rate for funeral ceremony in Tana Toraja, since the bulls are strongly related and important for Toraja culture. This situation cause the price of spotted buffalo, especially the bulls, is ten times above the normal buffalo.

Based on the usefulness in the tradition, spotted bulls are classified into different spotted types, *i.e Saleko, Bonga,* and *Lotong Boko* (Table 1 and Figure 1). There is no scientific and empirical explanation about the classification of spotted buffalo in correlation to the level of inbreeding. It is also not known if the classification is only based on qualitative variation. This study is a preliminary of an integrated research in order to answer that question. From conservation point of view, this classification makes easier for us to perform a study about genetic variation for biodiversity and cultural conservation purposes due to the availability of phenotypic data in detail.

Table 1. The classification of spotted and normal swamp buffalo based on coat and iris color (http://doddyg.blogspot.com.es/2013/05/wara-wiri-kerbau-toraja.html).

Coat color type	Coat color pattern	Iris color
Saleko	Black spot pattern that spread out on the white/pink-based coat on the body surface.	White
Bonga	The white/pink spot mostly on face, neck and nape, on the black-based coat color. Sometimes the spot could also find on the tail and legs.	White
Lotong Boko	Black spot on the white/pink-based coat color, especially on the back. Sometimes the black spot could also find on their face.	White
Solid	A normal swamp type has black/dark grey coat color, and 2 or 3 white lines on the neck, looks like a necklace.	Black

Figure 1. The classification of spotted and normal swamp buffalo based on the phenotype of coat and iris color, A: *Saleko*; B: *Bonga*; C: *Lotong Boko*; and D: Solid.

It is known that several genes as *TYR, TYRP1, DCT, MC1R, KIT, KIT ligand, MITF, SOX10, PAX3, EDNRB, ASIP, and PMEL17*, play important roles in pigmentation in other species (Hirooka et al. 2002; Royo et al. 2005; Hèdan et al. 2006; Karlsson et al. 2007; Mohanty et al. 2008; Stachurska and Brodacki 2008; Bauer et al. 2009; Fang et al. 2009; Karlsson et al. 2011). *KIT* gene known play important key roles in melanogenesis, erythropoiesis, spermatogenesis and T-cell differentiation (Yoshida et al. 2001). Based on this information, *KIT* could be used as one of candidate gene that bring the causative mutation for spotted phenotype.

The limitation of available data of spotted buffalo reproduction potency should not be an obstacle to starting the study. Even though it is not possible to collect ejaculated sperm due to cultural perspective; assisted reproductive technology (ART) can be a possible alternative to collect sperm from other sources. In this study we are going to collect sperm from cauda epididymis tissues of the slaughtered bulls. Our study was aimed to investigate the possibility and potency of using cauda epididymal sperm in spotted buffalo conservation program.

Classification of coat color pattern in spotted buffalo

Epididymal sperm from four bulls each from *Saleko, Bonga* and *Lotong Boko* types and from five solid bulls as control were used in this study. The age of the bulls were varied from six to ten years old.

Epididymal sperm collection

Epididymis tissues were collected in different time and ceremonies. Nevertheless, all bulls and samples got similar treatments during pre-slaughtered. Cauda epididymis was separated from other tissues (testis, ligament, scrotum, etc.) after an hour of transportation to the laboratory. Epididymal sperm was collected using incision method into a soya-lecithin based extender (Lone et al. 2011). Collected-sperm mix-solution was centrifuged at 500 g for 20 minutes to separate the sperm cells and debris. Pellet that contain sperm cells were evaluated for cell concentration, progressive motility, livability, normal morphology, and membrane integrity. Due to the high density of sperm cells in cauda epididymis and the limitation of tools that was available, the pellet was diluted using the soya lecithin-based extender up to 4 ml before it was counted manually. Good quality epididymal sperm suspensions were diluted in the same extender into 60.10^6 cell/ml and packed in 0.25 ml plastic straws.

Freezing and thawing

Straws were equilibrated at 4°C for 3 hours before freezing. The freezing process was started by placing the straws 10 cm above liquid nitrogen for 15 minutes and followed by plunging the straw into liquid nitrogen (-196°C) for storage. The frozen sperm were thawed at 37°C for 30 seconds.

Sperm evaluation

All parameters that evaluated were referred to Yulnawati et al. (2010). Progressive motility, livability and membrane integrity were evaluated from frozen-thawed semen samples randomly. Progressive motility was observed subjectively using a phase contrast microscope (Nikon, Japan) in 10 fields of view. Sperm livability was analyzed using the eosin-nigrosin staining method. Eosin cannot penetrate through an intact sperm membrane, with the result that living sperm do not stain whereas dead or dying sperm appear red against the blue background. Furthermore, membrane integrity was observed using the hypo-osmotic swelling test. Sperm samples were incubated for 30-45 minutes in hypo-osmotic medium. Sperm with an intact membrane have a swollen tail, while sperm with a damaged membrane have a straight tail when observed by phase contrast microscope.

Statistical analysis

Data from spotted individuals, which was classified into three groups, were compared to solid group as control. Each individual was considered as a repetition. Results were expressed as the means ± standard error mean (SEM). A difference with value $P<0.05$ was considered statistically significant.The data obtained were analyzed by one-way analysis of variance for comparison between each spotted versus solid group the help of Statistical Product and Software Solutionversion-13 (SPSS Inc., Chicago, IL, USA).

RESULTS

The average quality of fresh epididymal sperm from those three groups was not different compare to control group (Table 2). The fresh epididymal sperm quality from all groups was also qualified referred to Indonesian national standard requirement for buffalo frozen semen (SNI 01-4869.2-1998). The fresh epididymal sperm then was processed to be storage in the liquid nitrogen.

Table 2. Fresh epididymal sperm quality collected from different source of spotted buffaloes

Parameters observed	*Saleko*	*Bonga*	*LB*	Solid
Sperm concentration (10^6 cell/ml)	2,276 ± 546.4	2,442 ± 539.9	2,486 ± 718.9	2,544 ± 815.8
Progressive motility (%)	73 ± 2.5	73 ± 2.5	71.0 ± 2.0	72 ± 2.5
Livability (%)	85.8 ± 2.9	84.0 ± 1.8	82.8 ± 1.9	82.2 ± 1.5
Abnormality (%)	7.5 ± 0.9	6.8 ± 0.8	7.2 ± 0.7	7.2 ± 0.5
Membrane intactness (%)	86.2 ± 3.2	85.4 ± 2.2	84.2 ± 1.6	83.2 ± 1.7

No statistically different between groups were observed; LB: *lotong boko*.

Table 3. Frozen-thawed epididymal sperm quality of different spotted buffaloes

Parameters observed	*Saleko*	*Bonga*	*LB*	Solid
Progressive motility (%)	44 ± 2	42 ± 2.5	40 ± 3.2	42 ± 2.5
Livability (%)	64.9 ± 2.5	65.2 ± 1.9	62.6 ± 1.1	62.7 ± 1.9
Membrane intactness (%)	64.6 ± 2.5	67.1 ± 1.6	64.5 ± 1.7	64.1 ± 2.4

No statistically different between groups were observed; LB: *lotong boko*.

The frozen-thawed epididymal sperm quality that evaluated from spotted groups did not show significant differences compare to control group (Table 3). Data also showed that the frozen-thawed epididymal sperm was suitable to be used in AI program, with progressive motility more than 40% (Yulnawati et al. 2010). Due to Indonesian national standard value, the minimum percentage of progressive motility for buffalo sperm is 30% (SNI 4869.2-2008).

DISCUSSION

The progressive motility of both fresh and frozen sperm was similar in control and other groups, as well as other parameters ($P > 0.05$). Previous study showed that the progressive motility of frozen-thawed epididymal sperm of spotted buffalo was 41.67% (Yulnawati et al. 2010). Moreover, the percentage of livability of frozen-thawed epididymal sperm from three spotted groups and control (64.9%; 65.2%; 62.6% and 62.7%) in this study was also higher than previous study (52.2%) on the same species (Yulnawati et al. 2009). The percentage of membrane integrity is one of the important parameter that strongly related to the motility and fertility of the sperm. In this study, we obtained the results that showed the percentage of membrane integrity among three group of spotted bull was similar ($P>0.05$) to the control group. In general, the results showed that spotted coat color classification did not affect the quality, either fresh or frozen-thawed epididymal sperm.

This data showed that epididymal sperm of spotted bulls has good potency to be used widely in artificial insemination (AI) and other assisted reproductive technology (ART) application to increase population, as similar to the previous results in stallion (Morris et al. 2002), African buffalo (Herold et al. 2004), Iberian red deer (Martínez-Pastor et al. 2006), bull (Martins et al. 2009), ram (Lone et al. 2012; Álvarez et al. 2012). Nevertheless, the coat and iris color of the offspring is still cannot be predicted even though the epididymal sperm from spotted bulls are used in AI. Previous study showed variations of the offspring coat color from AI program (Said and Tappa 2008). This unclear situation suggests us to continue a genetic study to specify a good candidate gene(s) that contain the causative mutation(s) for spotted coat and white iris color.

As it is already mentioned in the introduction of this study, it is also known that KIT and KIT ligand were the strong candidate genes for the different degree of spotted expression on the coat color of mice (Ray et al. 1991), pig (Giuffra et al. 2002), Holstein cattle (Fontanessi et al. 2010), and caused the reduce of male fertility and also lead the abnormality during spermatogenesis (Phung et al. 2011). The precursor of upstream mechanism of action of those both genes is *MITF* gene (Phung et al. 2011; Phung et al. 2012). Since it shown in our results that there was no influence of spotted coat color to sperm quality, we suspect that the spotted pattern on Toraja buffalo coat color does not have any important mutation in the *KIT* and *KIT ligand* gene. On the other hands, it is known in other species that SNPs and mutation in *MITF* gene can cause the

spotted coat color in mice (Steingrimsson et al. 2004), dogs (Karlsson et al. 2007), white coat in German Fleikiech cattle (Phillip et al. 2011), and also splashed white in horse (Hauswirth et al. 2012). Furthermore, SNPs in *MITF* gene were also known as causative mutations that express micropthalmia in Waadenburg syndrome (Steingrimsson et al. 2004), dogs (Karlsson et al. 2007), albinism-deafness (Tietz) syndrome in human (Izumi et al. 2008), and deafness in cattle (Phillip et al. 2011). The investigation of mutation might be necessary for *MITF*, instead of *KIT* and *KITligand* gene due to the coat and iris color phenotype. The identification of causative mutation for spotted coat color is important to arrange a breeding plan to increase spotted buffalo population in a relative short time.

CONCLUSION

We conclude that the quality of fresh and frozen-thawed sperm of different spotted types buffalo are the same and it is good to be used in AI program.

REFERENCES

Álvarez M, Tamayo-Canul J, Martínez-Rodríguez C, López-Urueña E, Gomes-Alves S, Anel L, Martinez-Pastor F, de Paz P. 2012. Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). Anim Reprod Sci. 132:145-154.

Bauer GL, Praetorius C, Bergsteinsdóttir K, Hallsson JH, Gísladóttir BK, Schepsky A, Swing DA, O'Sullivan TN, Arnheiter H, Bismuth K, Debbache J, Fletcher C, Warming S, Copeland NG, Jenkins NA, Steingrímsson E. 2009. The role of *MITF* phosphorylation sites during coat color and eye development in mice analyzed by bacterial artificial chromosome transgene. Genetics. 183:581-594.

Fang M, Larson G, Ribeiro HS, Li N, Andersson L. 2009. Contrasting mode of evolution at a coat color locus in wild and domestic pigs. PLoS Genet. Vol. 5 Issue 1. www.ncbi.nlm.nih.gov/pubmed/19148282.

Fontanessi L, Tazzoli M, Russo V, Beever J. 2010. Genetic heterogeneity at the bovine KIT gene in cattle breeds carrying different putative alleles at the spotting locus. Anim Gen. 41:295-303.

Giuffra E, Törnsten A, Marklund S, Bongcam-Rudlof E, Chardon P, Kijas JMH, Andersson SI, Archibald AL, Andersson L. 2002. A large duplication associated with dominant white color in pigs originated by homologous recombination between LINE elements flanking KIT. Mamm Genome. 13:569-577.

Hauswirth R, Haase B, BlatterM, Brooks SA, Burger D, Dröemüller C, Gerber V, Henke D, Janda J, Jude R, Magdesian KG, Matthews JM, Poncet PA, Svansson V, Tozaki T, Wilkinson-White L, Penedo MCT, Rieder S, Leeb T. 2012. Mutations in *MITF* and *PAX3* cause ‘‘splashed white’’ and other white spotting phenotypes in horses. PLoS Genet. Vol. 8 Issue 4. http://www.ncbi.nlm.nih.gov/pubmed/?term=Hauswirth.

Herold FC, Aurich JE, Gerber D. 2004. Epididymal sperm from the African buffalo (*Syncerus caffer*) can be frozen successfully with Andromed® and Triladyl™ but the addition of bovine seminal plasma is detrimental. Theriogenology. 61:715-724.

Hèdan B, Corre S, Hitte C, Drèano S, Vilboux T, Derrien T, Denis B, Galibert F, Galibert M, Andrè C. 2006. Coat colour in dogs: identification of the *Merle* locus in the Australian shepherd breed. BMC Vet Res. Vol. 2: issue 9. http://www.biomedcentral.com/1746-6148/2/9.

Hirooka H, de Koning DJ, van Arendonk JAM, Harlizius B, de Groot PN, Bovenhuis H. 2002. Genome scan reveals new coat color loci in exotic pig cross. J Hered. 93:1-8.

Izumi K, Kohta T, Kimura Y, Ishida S, Takahashi T, Ishiko A, Kosaki K. 2008. Tietz syndrome: unique phenotype specific to mutations of *MITF* nuclear localization signal. Clin Genet. 74:93-95.

Karlsson AC, Mormede P, Kerje S, Jensen P. 2011. Genotype on the pigmentation regulating PMEL17 gene affects behavior chickens raised without physical contact with conspecifics. Behav Genet. 41:312-322.

Karlsson EK, Baranowska I, Wade CM, Hillbertz NHCS, Zody MC, Anderson N, Biagi TM, Patterson N, Pielberg GR, Kulbokasiii EJ, Comstock KE, Keller ET, Mesirov JP, von Euler H, Kampe O, Hedhammar A, Lander ES, Andersson G, Andersson L, Lindbald-Toh K. 2007. Efficient mapping of mendelian traits in dogs through genome-wide association. Nat Genet. 39:1321-1328.

Lone FA, Islam R, Khan MZ, Sofi KA. 2011. Effect of transportation temperature on the quality of caudaepididymal spermatozoa of ram. Anim Reprod Sci. 123:54-59.

Martins CF, Driessen K, Melocosta P, Carvalho-Neto JO, V. de Sousa R, Rumpf R, Dode MN. 2009. Recovery, cryopreservation and fertilization potential of bovine spermatozoa obtained from epididymides stored at 5ºC by different periods of time. Anim Reprod Sci. 116:50-57.

Martinez-Pastor F, Martinez F, Garcia-Macias V, Esteso MC, Anel E, Fernandez-Santos MR, Soler AJ, de Paz P, Garde J, Anel L. 2006. A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology. 66:1165-1172.

Mohanty TR, Seo KS, Park KM, Choi TJ, Choe HS, Baik DH, Hwang IH. 2008. Molecular variation in pigmentation genes contributing to coat colour in native Korean Hanwoo cattle. Anim Genet. 39:550-553.

Morris L, Tiplady C, Allen WR. 2002. The in vivo fertility of cauda epididymal spermatozoa in the horse. Theriogenology. 58:643-646.

Phillip U, Lupp B, Mömke S, Stein V, Tipold A, Eule JC, Rehage J, Distl O. 2011. A MITF mutation associated with a dominant white phenotype and bilateral deafness in German Fleckvieh cattle. PLoS One. Vol. 6 Issue 12. http://www.ncbi.nlm.nih.gov/pubmed/22174915.

Phung B, Sun J, Schepsky A, Steingrimsson E, Rönnstrand L. 2011. c-KIT signaling depends on microphthalmia-associated transcription factor for effects on cell proliferation. PLoS One. Vol. 6 Issue 8. http://www.ncbi.nlm.nih.gov/pubmed/?term=Phung.

Phung B, Sun J, Schepsky A, Steingrimsson E, Rönnstrand L. 2012. Elucidation of c-KIT-dependent signaling to *MITF*. Cancer Res: 72 (Suppl 1).

Ray P, Higgins KM, Tan JC, Chu TY, Yee NS, Nguyen H, Lacy E, Besmer P. 1991. Ectopic expression of a c-*kit*^{w42} mini-gene in transgenic mice: recapitulation of W phenotypes and evidence for c-*kit* function in melanoblast progenitors. Genes Develop. 5:2265-2273.

Royo LJ, Alvarez IA, Fernandez I, Arranz JJ, Gomez E, Goyache F. 2005. The coding sequence of the *ASIP* gene is identical in nine wild-type coloured cattle breeds. J Anim Breed Genet. 122:357-360.

Said S, Tappa B. 2008. Perkembangan kerbau belang (*Tedong bonga*) di Puslit Bioteknologi LIPI Cibinong, Jawa Barat dengan teknologi reproduksi. Proceeding of National Seminar for Buffalo Breeding. Tanah Toraja, 24-26 2008. Tanah Toraja (Indones). Pusat Penelitian dan Pengembangan Peternakan. p. 18-25.

Stachurska A, Brodacki A. 2008. Variation of gene frequencies in ASIP, MC1R and GREY loci in thoroughbred horses. Livestock Sci. 113:163-168.

Steingrimsson E, Copeland NG, Jenkins NA. 2004. Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet. 38:365-411.

Yoshida H, Kunisada T, Grimm T, Nishimura EK, Nishioka E, Nishikawa SI. 2001. Review: melanocyte migration and survival controlled by SCF/c-kit expression. J Investig Dermatol Symp Proc. 6:1-5.

Yulnawati, Gunawan M, Maheshwari H, Rizal M, Herdis, Boediono A. 2010. Quality of epididymal and ejaculated sperms of spotted buffalo in dextrose supplemented extender. Hayati J Bioscience. 17:27-30.

Yulnawati, Maheshwari H, Herdis, Rizal M. 2009. Viability and plasma membrane integrity of the spotted buffalo epididymal spermatozoa after thawing in the addition of dextrose into the extender. Biotropia. 16:20-26.