Spermatogenesis and semen quality of male muntjak *(Muntiacus muntjak muntjak)* during antler growth periods

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Abstract. Muntjak *(Muntiacus muntjak muntjak)* belongs to Cervidae family which distributed in Java Island and Southern part of Sumatera. This cervid has been protected by Indonesian Government since 1999. In order to support breeding program of the species and to avoid them from extinction, its reproductive biology such as spermatogenesis and the correlation to semen quality is important to be investigated. Therefore, the objective of this study was to examine spermatogenesis and semen quality of two adult male muntjaks during antler growth periods that consist of hard antler (H), casting (C), and velvet antler (V). Testicular tissues and semen (ejaculates) were obtained by core needle biopsy and electroejaculation methods respectively. Testicular tissues were processed histologically and stained with periodic acid Schiff (PAS) to observe spermatogenesis whereas semen was evaluated to obtaine its quality. The results showed that spermatogenic activities were detected in all antler periods which marked by PAS positive staining (magenta colour) of round and elongated spermatid acrosomes. In H period, spermatogenic activity was higher than those C and V periods. According to semen evaluation, motile spermatozoa were found with different concentration in all antler periods. The highest sperm concentration (x 10^6 spermatozoa/ml) in both of muntjaks was found in H period (506.25 ± 61.87), and slightly decreased in C (288.75 ± 37.12), and V periods (362.60 ± 17.68). These finding showed that spermatogenesis to produce spermatozoa is taken place while muntjaks are in C and V periods with differ activities that provable with the existence of motile spermatozoa from ejaculates in both of male muntjaks. Therefore, muntjaks could provide reproductive function throughout the year of reproductive aseasonality which is similar to the reeves and formosan muntjaks.
Keywords: spermatogenesis, antler growth periods, male muntjak, spermatozoa

Introduction

Muntjak *(Muntiacus muntjak spp)* or barking deer is one of deer species. In Indonesia, this cervid is known as kijang with six sub species, i.e. *M. m. muntjak* (Java and Southern part of Sumatera), *M. m. montanus* (North Sumatera), *M. m. bancanus* (Bangka and Belitung), *M. m. nainggolani* (Bali and Lombok), *M. m. pleicharicus* (Borneo, Matasiri, Bawal, and Java), and *M. m. robinsoni* (Riau and Lingga Island) (Maryanto *et al* 2008). Because of decreasing in number, the entire sub species have been protected by Indonesian Government since 1999 (PHKA 2004). The number of muntjak in both wild and captivity in Indonesia is unknown exactly. Therefore, to support breeding program and avoid muntjak from extinction, the reproductive biology of the species was important to be investigated.

There is variation in reproductive pattern of male and female cervids that living in temperate and tropical region. Cervids or deer living in temperate latitude show seasonal pattern in their reproductive activities (Li *et al*. 2001). Therefore, there are invariably infertile during non breeding season or in antler growing period (Asher *et al*. 2000). During breeding season when day length decreased, reproductive activities are very conspicuous. In contrast, most tropical cervids are aseasonal in their calving pattern. Although some individual male cervids show an annual antler cycles, viable spermatozoa have been collected in all antler periods such as axis deer, *Axis axis* (Loudon and Curlewis 1988). Other cervid species that aseasonal reproductive is timor deer *(Cervus timorensis)*, however, good quality of semen in this deer only occurred in hard antler and decreased in casting and velvet antler periods (Handarini *et al*. 2004). Other species of muntjaks i.e. reeves muntjak *(M. reevesi)* and formosan muntjak *(M. m. micrurus)* are also aseasonal but then they reproductive activities still taken place although they are in casting and velvet periods (Chapman and Harris 1991; Pei *et al*. 2009).

Spermatogenesis is a complex process of cells division and cells differentiated to produce spermatozoa that occur in seminiferous tubules of the testis. Spermatogenesis comprises three events that is spermatocytogenesis, meiosis, and spermiogenesis which are characterized by spermatogonia, spermatocytes, and spermatids (round and elongated), respectively (Johnson *et al*. 2000). This process can be detected by using histochemistry procedure as periodic acid Schiff (PAS) staining. In this staining, acrosomes cap on the surface of the round and elongated spermatids will positively react with PAS (Dreff *et al*. 2007).

In order to know the level of spermatogenesis in each antler stage, those staining procedure could provide a valuable data and subsequently provable with semen quality. Some microscopic parameters of semen quality are sperm concentration, motility, and life and abnormality of spermatozoa. Detection on spermatogenesis in cervids has been applied in roe deer (Blottner *et al*. 1996; Goeritz *et al*. 2003), mule deer (Heckmann 2009) whereas semen quality has been reported in red deer (Gizejewski *et al*. 2010), timor deer (Nalley *et al*. 2012).

Reproductive pattern of male muntjak *(M.m. muntjak),* however, remain unclear. Therefore, understanding reproductive capability of this species, particularly concerning spermatogenic activity and semen quality was important to be examined. In the present study, we investigated spermatogenesis and its relation to semen quality during antler growth periods. These findings can support breeding program of muntjak in captivity.

Materials and Methods

Animal

Testicular tissue and semen were collected from two adult and healthy male muntjaks (*Muntiacus muntjak muntjak)* aged 2-4 years old; 17-19 kg body weights. These Cervids were obtained from Central Java, Indonesia under permission of SK 23/Menhut-II 2012 from Indonesian Ministry of Forestry, Republic of Indonesia. Muntjaks were housed individually and connected to outdoor enclosure and maintained within visual and olfactory proximity to female muntjaks. Both of samples: semen or ejaculates and testicular tissue were collected once in hard antler (H), casting or detached antler (C), and velvet (V) periods. Muntjaks were immobilized using a combination of xylazine HCl and ketamin intra muscularly (i.m) prior testicular biopsy and semen collection procedures.

Semen collection and evaluation

Semen was collected before biopsy procedure using electroejaculation method as described by Okano *et al*. (2004) with some modification. We used an electroejaculator (Fujihira, FHK, Japan) with 100 Hz AC stimulator that equipped with four circular electrodes around a rectal probe. Probe was inserted 10 cm into rectum and stimulated by electric current (range 3 to 7 volts) and each stimulus lasted 5 seconds with subsequent pause for 10 seconds. The penis was washed with sterile physiological saline. After emission, erection, and ejaculation, semen was forced into a sterile glass tube. Semen was evaluated to assess sperm concentration, motility, life and, abnormality. Sperm concentration was estimated by haemocytometer and Neubauer chamber. Subjective observation was applied to know sperm motility and scored into 0-100%. In addition, life and abnormality sperm was observed by 2% eosin staining on slide containing semen and observed under a light microscope.

Testicular biopsy

Prior biopsy using core needle biopsy method (CNB), the surface of scrotal skin of the testis was antisepticised using ethanol 70% and iodine. Biopsy method was performed as described previously by Tuuri *et al*. (1998). Biopsy was taken in dorsal area of right or left testis. A 14 *gouce* needle (Fine core 'Toray' 14 G, Japan) was inserted through scrotal skin to the testicular tissues. Once needle was inside of testis, a strong negative pressure of biopsy gun was exerted for cut and obtained testicular tissue. After tissues aspiration, the needle was withdrawn slowly from the testis and scrotal skin. The area of scrotal skin after biopsy gun injected was treated by antibiotic ointment topically and injected by long acting antibiotic intra muscularly.

Histological preparation

Testicular tissue which was obtained by CNB method subsequently fixed immediately in Bouin's fixative for 24 h and transferred to ethanol 70% for stopping point. The samples were dehydrated in a graded series of ethanol, clearance in xylane, paraffin infiltrated and embedded in paraffin. Samples in paraffin were cut at 2-3 µm of thickness using sliding microtome and stained with periodic acid Schiff (PAS). The stained section were examined under a light microscope and photographed by digital camera. Positive reaction was marked by presence of magenta color in acrosomal of round and elongated spermatids.

Results and Discussion

Semen quality

The results of semen evaluation from two adult male muntjaks in all of antler growth (H, C, and V) periods are presented in Table 1. In general, good quality of semen in those male muntjaks was found in H period than C and V periods. However, the highest sperm concentration (x 10^6 spermatozoa/ml) was found in H period (506.25 ± 61.87), and slightly decreased in C (288.75 ± 37.12), and V periods (362.60 ± 17.68). The amount of sperm in C and V periods, however, is fairly well than timor deer (Handarini *et al*. 2004). At the same periods (C and V), sperm concentration of timor deer was decreased drastically and lower than its concentration in muntjak. Good quality of muntjak's semen was also indicated by highly percentage of motility and life sperm, and also low sperm abnormality, i.e. 70% and 91.5 ± 0.77% and 6.7 ± 0.7% respectively. Sperm abnormality slightly increased in V period (13.0 ± 0.7%) but then the amount is lower than sperm abnormality in timor deer at the same period (35.59 ± 19.97%). Based on those results, the pattern of sperm production in muntjaks differs with timor deer where semen quality still good although muntjak be in C and V periods.

Table 1. Semen quality of two adult male muntjaks during antler growth periods.

Semen quality	Antler growth periods		
	H	C	V
Motility (%)	60	60	70
Concentration (x 10^6/ml)	506.25 ± 61.87	288.75 ± 37.12	362.6 ± 17.67
Life sperm (%)	91.35 ± 0.77	87.1 ± 0.7	89.0 ± 0.7
Abnormality (%)	6.7 ± 0.7	13.0 ± 0.7	12.45 ± 2.19

Good quality of muntjak's semen than timor deer may caused by variation in level of male steroid hormone (testosterone). Testosterone plays a central role in spermatogenesis to produce spermatozoa. Other role of testosterone is as regulator on antler growth. The peak of testosterone secretion lasted during H period and reach the basal level when cervids experience antler cast (casting) and during antler growth (velvet antler). This phenomenon clearly showed in most cervids in temperate region such as Iberian red deer (Malo *et al*. 2009) and also timor deer that distributed in Indonesia (Handarini & Nalley 2008). In muntjak *(M.m. muntjak),* highly level of testosterone was found during H period. However, this steroid hormone is still detected in sufficient concentration during C and V periods (Wahyuni 2012). These findings refer a close correlation between semen quality and level of testosterone during antler growth period.

Spermatogenesis

Microscopic examination on spermatogenesis activity that observed in three antler growth periods is presented in Figure 1 and Table 2. In all antler growth periods, the round and elongated spermatids were observed with variation in number. Positive reaction of PAS staining clearly showed in H period with highly intensity of magenta color in acrosome cap of round and early elongated spermatids. Appearance of magenta color in acrosome indicated that acrosome containing glycoprotein which can be visualized by PAS staining (Dreef *et al*. 2007). The number of positive reaction and also it color intensity was reduce in C and V periods. However, the feature of differentiation of spermatid during spermiogenesis still observed while muntjaks in C and V periods. The presence of round and elongated spermatid in entire antler growth periods indicates continuing of spermatogenesis to produce

spermatozoa. This finding showed a close correlation between sperm quality and spermatogenic activity during antler growth periods.

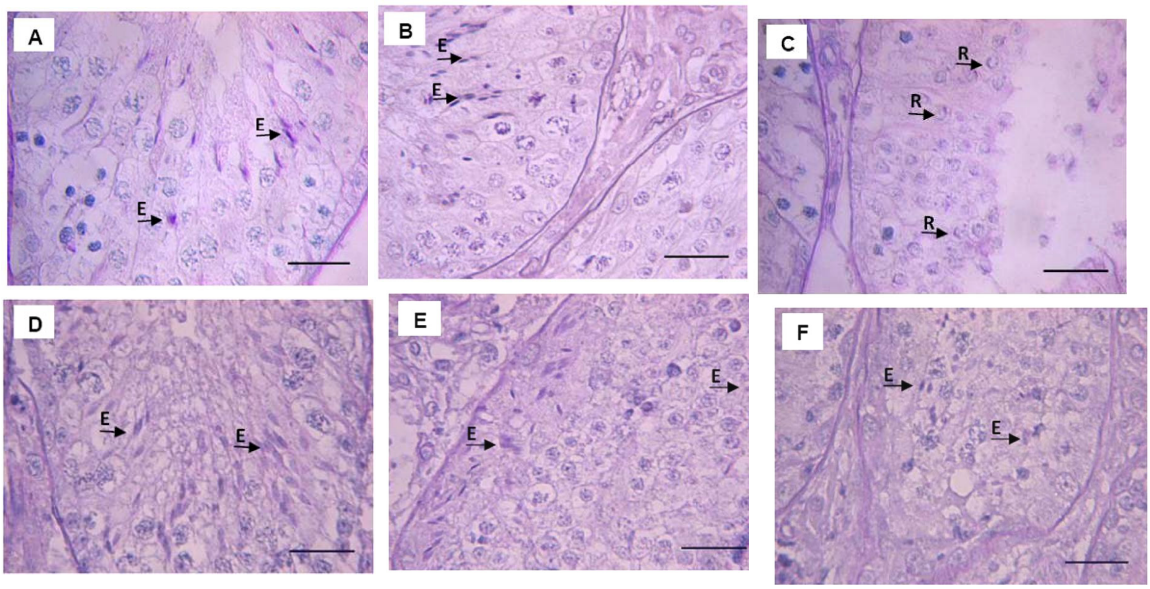

Figure 1. Cross section of seminiferous tubules of the muntjak's testis showed spermatogenesis in all antler growth periods. Appearance of spermatids during hard antler (A, B, C), casting (D), and velvet (E, F) periods. Positive reaction of PAS is indicated by magenta color (black arrow). Round spermatids (R), and elongated spermatids (E). Periodic acid Schiff (PAS) staining. Bar scale 30 µm.

Conclusions

Reproductive pattern of male muntjak is aseasonal that could provide reproductive function throughout the year. These findings could support captive breeding program of muntjak in Indonesia.

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