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MORPHOLOGICAL AND LECTIN HISTOCHEMICAL STUDIES OF THE SPERMATOZOA OF THE LESSER MOUSE DEER (*Tragulus javanicus*)

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SUMMARY

The morphology and lectin histochemical property of the spermatozoa of the lesser mouse deer (*Tragulus javanicus*) were studied using light and scanning electron microscopy. Spermatozoa were small in size and measured 36.52 ± 5.6 μm in length. The head of the spermatozoa was relatively round in shape (5.55 ± 0.8 μm in length and 4.77 ± 0.5 μm in width). Lectin peanut (*Arachis hypogea*) agglutinin (PNA) and lectin wheat germ (*Triticum vulgaris*) agglutinin (WGA) were positive in the membrane of the acrosomal region, and in the membrane at the anterior part of the acrosomal region and in the tail, respectively. It is suggested that carbohydrates with galactose β1-3, D-N acetylgalactosamine, D-N acetylglucosamine and sialic acid sugar residues may be involved and may have significant roles in the spermatozoa in the lesser mouse deer.

Keywords: Sperm morphology, lectin histochemistry, lesser mouse deer, scanning electron microscopy

INTRODUCTION

There is considerable variation in the size and shape of the head of the spermatozoa among species. For example, it is columnar and elongated in fowl, like a hook in the mouse and rat, or flattened and ovoid in man (Mann, 1964). The entire spermatozoon is covered by plasma membrane, which is rich in carbohydrate molecules. The majority of sugar residues are attached to the sperm membrane proteins. These glycoproteins may be closely related with sperm maturation, protection within the female genital tract, binding of spermatozoa to the zona pellucida and with the fertilisation process(Gilbert, 1988; Nolan and Hammerstedt, 1997; Zara and Naz, 1998; Tulsiani, 2000).

The lesser mouse deer (*Tragulus javanicus*) is regarded as the smallest ruminant in the world (Lekagul and McNeely, 1977) and is an ideal model in ruminant and biomedical research (Sastradipradja, 1978). However, there is a paucity of information on its reproductive biology. In previous studies, the characteristics and quality (Haron *et al.*, 1999) and chemical composition (Prasetyaningtyas *et al.*, 2006) of the sperm of the lesser mouse deer obtained by electroejaculation were reported. However, the sperm morphology and histochemical properties regarding the distribution of specific carbohydrates have not been studied in the lesser mouse deer.

In the present study, the morphology and the distribution of lectin peanut (*Arachis hyppogea*) agglutinin (PNA) and wheat germ (*Triticum vulgaris*) agglutinin (WGA) bindings in the spermatozoa of the lesser mouse deer (*Tragulus javanicus*) were investigated in order to provide information on the reproductive biology of this species.

MATERIALS AND METHODS

Semen collection

Four young adult male lesser mouse deer weighing 1.8 – 2.4 kg were used in this study. The age of the animal was estimated to be around 12-18 months based on the appearance of canine teeth. The animals were housed in the animal facility at the Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia. Each animal was placed in an individual cage measuring 1 x 2

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m^2. They were fed twice a day with vegetables which included *ipomoea* leaves, long beans, lettuce, carrots, and potatoes, and fruits such as bananas and apples according to Jumaliah (1999) and water was given *ad libitum*.

The semen samples were collected twice a month for a duration of one year, using an electro-ejaculator (Electric Stimulator, Fujihiro Industry Co., Ltd, Tokyo, Japan) after the animals were anaesthetised using a combination of xylazine (Xylazyl 100®, Troy Lab Pty., Ltd., NSW, Australia; 0.1mg/kg BW) and ketamine (Ketavet 100®, Delvet Pty., Ltd., NSW, Australia; 11mg/kg BW). The electrical stimulations at low frequency were applied intermittently (Axner and Linde-Forsbeg, 2002) at 5-15 volts for a duration of 5 seconds, followed by 2-3 seconds rest. A total of 16 semen samples were observed in this study.

Morphological studies

One drop of the semen sample was placed onto the object glass to which a cover slip was mounted to make native specimens. Other semen samples were processed for scanning electron microscopy (SEM). Briefly, a semen sample was dropped onto a cover glass and air dried. The samples were fixed in 2.5% glutaradehyde for 2 h and were then immersed in 2% tannic acid for 1 h (Murakami, 1974). After washing with 0.01M PBS pH 7.4, the samples were then post fixed using 1% osmium tetroxide (OsO_4) for 1 h, dehydrated in graded series of alcohol and *t*-butanol (Inoue and Osatake, 1988) and freeze dried using a freeze dryer (VFD-2ls, Tokyo, Japan). Samples were then coated with platinum-palladium using an ion coater (Eiko IB-3, Tokyo, Japan) and observed using a SEM (JEOL, LV-5800, Tokyo, Japan) at magnification of 1000-2000x and an accelerating voltage of 10 kV. The measurement of the spermatozoa was taken on native specimens using a light microscope equipped with measuring gauge and also from the SEM images. In the light microscopy, the measurement was taken at 400x magnification from around 50 spermatozoa per specimen. The length and width of the sperm head and also the total length of the spermatozoa were recorded in the spermatozoa with normal morphology.

Lectin histochemistry

Semen samples were centrifuged at 640xg for 5 min and the sediment was used to make smear specimens. The specimens were fixed in 2.5% glutaraldehyde for 30 min at 60°C and stained using peroxidase labeled PNA and WGA lectins (Honen Co., Tokyo, Japan). The details of the lectins used are shown in Table 1.

Briefly, after washing with PBS the specimens were treated with 3% H_20_2 in methanol for 10 min to block the activity of endogenous peroxidase. Specimens were then washed with PBS and stained with peroxidase labeled lectins (10μg/ml of PNA and 15μg/ml of WGA) for 2-3 h at 37°C. Positive reactions were visualised with 0.02% 3, 3'-diaminobenzidine hydrochloride (DAB, Dojindo, Tokyo, Japan) and 0.003% H_20_2 in Tris buffer.

RESULTS

The spermatozoa of the lesser mouse deer were small in size and measured 36.52 ± 5.6 μm in total length. The head of spermatozoa was relatively round, with 5.55 ± 0.8 µm in length and 4.77 ± 0.5 μm in width (Figure 1, Table 2). In the specimens stained by lectin histochemistry, lectin PNA and lectin WGA was positive in the acrosomal membrane (Figure 2a), and in the membrane of the anterior acrosomal region and tail (Figure 2b), repectively.

Table 1: Dilution and specificity of the lectin used

Taxonomic name	Acronym	Concentration	Specificity
Arachis hypogea	PNA	10 μg/ml	Galactose β1-3, D-N-acetylgalactosamine
Triticum vulgaris	WGA	15 μg/ml	D-N-acetylglucosamine, sialic acid

Table 2: Measurement of the spermatozoa of the lesser mouse deer

	Size (μm)
Total length	36.52 ± 5.6
Head	
Length	5.55 ± 0.8
Width	4.77 ± 0.5

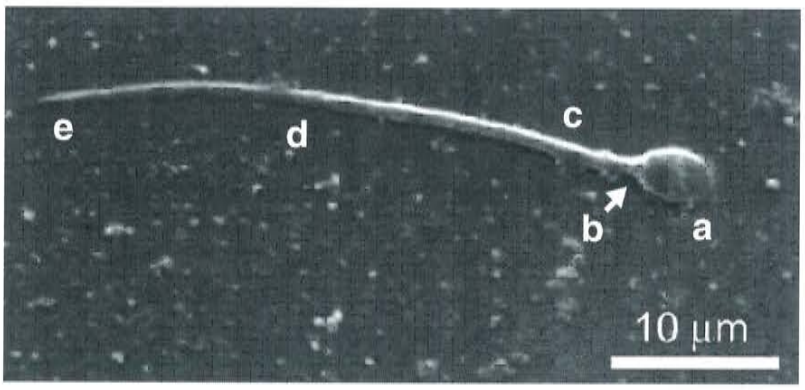

Figure 1: Photomicrograph of the spermatozoon of the lesser mouse deer. a. Head, b. Neck, c. Body, d. Principal tail, e. End tail. SEM. 10kV.

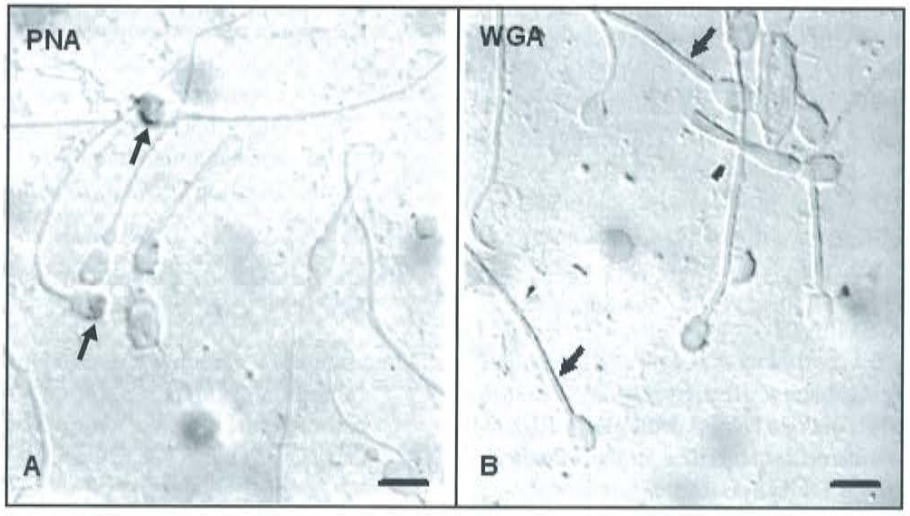

Figure 2: Distribution of lectin-bindings in the membrane of the spermatozoa of the lesser mouse deer. PNA (A) is positive in the acrosomal membrane (arrow), while WGA (B) is positive in the membrane of anterior acrosomal region (long arrow) and tail (short arrows). Peroxidase conjugated lectin. Scale bars: 5μm

DISCUSSION

The shape of the head of the spermatozoon in the lesser mouse deer is relatively more round when compared to the other ruminants (Mann, 1964; Bustos-Obregos and Fléchon, 1975; Salisbury and Van Demark 1985; Evans and Maxwell, 1987). Further, the spermatozoon of the lesser mouse deer was considerably smaller than the values reported in ram (Bustos-Obregon and Fléchon, 1975; Evans and Maxwell, 1987; Rizal, 2005), bull (Bustos-Obregos and Fléchon, 1975; Salisbury and Van Demark 1985) and boar (Bustos-Obregos and Fléchon, 1975).

Lectins are commonly used as a marker to assess carbohydrate composition in the sperm membrane (Bearer and Friend, 1990), sperm acrosomal status and acrosome reaction (Cheng *et al.*, 1996). Sperm with the intact acrosome will undergo acrosome reaction which is essential for sperm penetration and fusion and damages of the acrosome can decrease the fertility rate of the spermatozoa (Nolan and Hammerstedt, 1997; Hammadeh *et al.*, 2001; Ramalho-Santos *et al.*, 2002; Yoshinaga and Toshimori, 2003). This study demonstrated the presence of PNA bindings in the acrosomal membrane and WGA bindings on the membrane of anterior acrosomal region and tail of the spermatozoa of the lesser mouse deer. Lectin PNA indicated carbohydrates with galactose β1-3 and D-N-acetylgalactosamine sugar residues. These glycoproteins are closely related to various processes in the cell to cell recognition and adhesion of gametes cells, sperm and zona pellucida binding or binding of sperm and epithelia of the female reproductive organ (Gilbert, 1988; Spicer and Schulte, 1992; Thall *et al.* 1995; Clark *et al.*, 1996; Zara and Naz, 1998).

Lectin WGA indicated carbohydrates with D-N acetylglucosamine and sialic acid sugar residues. D-N acetylglucosamine residues function in the binding and fusion of sperm and oocytes (Gougoulidis *et al.*, 1999). Furthermore the N-acetylglucosaminidase (NAG), a

glycosidase-recognising N-acetylglucosamine terminal residue, is also involved in the primary binding to zona pellucida and penetration and polyspermy block (Zitta *et al.*, 2006). Sialic acid residues are secreted by the epithelium of the epididymis as terminal sugar of sialoglycoproteins. These glycoproteins bind to the sperm surface during epididymal transit (Lassale and Testart, 1994) and cover the spermatozoa surface and protect them from phagocytic activity by leukocyte and vaginal epithelial cells (Harayama *et al.*, 1996; Lassalle and Testart, 1994). Sialic acid may also participate in the binding of spermatozoa and zona pellucida in human (Ozgur *et al.*, 1998).

The PNA bindings in the spermatozoon of the lesser mouse deer are similar to those reported in ram (Flesch *et al.*, 1998), *Bufo arenarum* (Martinez and Cabada 1996), bull (Cross and Watson, 1994), human (Mortimer *et al.*, 1987) and stallion (Cheng *et al.*, 1996) while the WGA bindings are generally similar to those reported in human sperm (Lasselle and Testart, 1994; Gabriel *et al.*, 1995), and boar (Jimenéz *et al.*, 2003). This suggests that carbohydrates with galactose β1-3, D-N-acetyl-galactosamine, D-N-acetylglucosamine and sialic acid sugar residues are involved and may have significant roles in the spermatozoa of the lesser mouse deer.

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