Jurnal Kedokteran Hewan March 2022, 16(1):12-17
P-ISSN : 1978-225X; E-ISSN : 2502-5600 DOI: https://doi.org/10.21157/j.ked.hewan.v16i1.24418

CHARACTERIZATION OF GLYCOCUNJUGATE DISTRIBUTION IN THE EPIDIDYMIS OF THE JAVAN MUNTJACS (*Muntiacus muntjak muntjak*)

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ABSTRACT

The presence of glycoconjugates in caput, corpus, and cauda epididymis of the Javan muntjacs was performed using lectin histochemical method. The epididymis organs were obtained from two adult male Javan muntjacs in hard antler stage and then immediately fixed in Bouin's solution and processed in histological manner. Six types of biotinylated lectins namely Peanut agglutinin (PNA), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA), Ricinus communis agglutinin (RCA), Concanavalin A (Con A), and Ulex europaeus agglutinin I (UEA I) were applied to detect the specific glycoconjugates in the epididymal tissue of muntjacs. The result showed the presence of glycoconjugates were detected by five types of lectins except UEA I in caput, corpus, and cauda epididymis with specific distribution and also the intensity of lectins binding. Glycoconjugates β-galactose, β-glucose, mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, and sialic acid were stained with various intensity in epithelial cells, basal cells, stereocilia, spermatozoa, and stroma of caput, corpus, and cauda epididymis. The presence of sialic acid on the surface of spermatozoa detected by WGA are thought to be play a role on sperm protection during their maturation and transit along epididymal lumen. In conclusion, the difference of glycoconjugates distribution pattern and lectin binding in the epididymal caput, corpus, and cauda of the Javan muntjac indicates the regional differences in epididymal function in spermatozoa maturation.

Key words: epididymis, glycoconjugates, Javan muntjac, lectins binding

ABSTRAK

Keberadaan glikokonjugat pada kaput, korpus, dan kauda epididimis mucak jawa dideteksi menggunakan metode histokimia lektin. Organ epididimis diambil dari dua ekor muncak jawa jantan dewasa yang berada pada tahap ranggah keras kemudian difiksasi menggunakan larutan Bouin dan diproses secara histoteknik. Pada penelitian ini digunakan enam jenis lektin, yaitu Peanut agglutinin (PNA), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA), Ricinus communis agglutinin (RCA), Concanavalin A (Con A), and Ulex europaeus agglutinin I (UEA I) untuk mendeteksi glikokonjugat spesifik pada jaringan epididimis muncak. Hasil pengamatan menunjukkan keberadaan glikokonjugat pada bagian kaput, korpus, dan kauda epididimis yang bereaksi dengan lima jenis lektin kecuali UEA I dengan sebaran dan intensitas ikatan lektin yang spesifik. Glikokonjugat β-galaktosa, β-glukosa, manosa, N-asetil-galaktosamin, N-asetil-glukosamin, dan asam sialat terwarnai dengan intesitas bervariasi pada sel-sel epitel, sel basal, stereosilia, spermatozoa, dan stroma kaput, korpus, dan kauda epididimis. Keberadaan asam sialat pada permukaan spermatozoa yang terdeteksi dengan lektin WGA diduga berperan dalam melindungi spermatozoa selama proses maturasi dan perpindahan di sepanjang lumen epididimis. Kesimpulan dari penelitian ini adalah terdapat perbedaan pola sebaran glikokunjugat dan intensitas ikatan lektin pada bagian kaput, korpus, dan kauda epididimis muncak jawa mengindikasikan perbedaan fungsi setiap bagian epididimis dalam proses maturasi spermatozoa.

Kata kunci: epididymis, glikonjugat, muncak jawa, ikatan lektin

INTRODUCTION

Muntjac (*Muntiacus muntjak spp*) belongs to the Cervidae that is widely distributed in South East Asia including Indonesia. This species in Indonesia consists of several sub-species, namely *M. m. muntjak* (Java Island), *M. m. montanus* (Sumatra Island), *M. m. nainggolani* (Bali and Lombok Islands), *M. m. pleicharicus* (Borneo Island), and *M. m. robinsoni* (Bintan and Linga Islands) (Maryanto *et al*. 2008). However, all of these muntjac sub-species have been protected by the Indonesian Government since 1999. The protection status of muntjacs has been listed in the Attachment List to Government Regulation of the Republic of Indonesia Number 7, 1999 (PHKA 2004).

Various basic data on the reproductive biology of male muntjac is required to support the breeding programs of this species in Indonesia, especially the sub-species *M. m. muntjak.* Several aspects of the reproductive biology of *M. m. muntjak* known as Javan muntjac have been reported by Wahyuni *et al*. (2011), Wahyuni *et al*. (2012a), Wahyuni *et al*. (2012b), Wahyuni *et al*. (2013), Wahyuni *et al*. (2016), Wahyuni *et al*. (2018). Although several studies on that species, particularly regarding male reproductive organs have been carried out, the characterization of glycoconjugates distribution in the epididymis has not been reported.

Glycoconjugate is carbohydrate compounds that bind to proteins in the form of proteoglycans and glycoproteins, while those that bind to fats are known as glycolipids. These compounds are widely distributed in almost all body tissues, mainly on the surface and cytoplasm of cells as well as in the extracellular matrix (Kiernan 1990). The presence of glycoconjugates in tissues have many important roles, such as in the process of cell differentiation and maturation (Damjanov 1987), recognition and adhesion to cells

Revised 21-02-2022, Accepted 08-03-2022

(Sharon and Lis 1989), and cells interactions (Wassarman 1989). Glycoconjugates in the mammalian reproductive tract are very important components of the molecular mechanisms that control the maturation and transport of spermatozoa, and the interactions between male and female gamete cells. Furthermore, glycosylation in spermatozoa can be extensively modified during spermatozoa transport and maturation (Diekman 2003). Additionally, glycoproteins in epididymis tissue are highly glycosylated and the glycan portion seems play a role in sperm maturation (Srivastav *et al*. 2004).

Glycoconjugates content in tissue, both in cells and in the matrix can be detected by histochemical method using several lectins. This method has been widely applicated in animal tissue to recognize specific glycoconjugates based on the affinity of sugar residues from carbohydrates (Kiernan 1990). Furthermore, detection of cellular glycoconjugates by protein (lectin)-carbohydrate interaction is not only valuable to delineate characteristic changes in glycosylation of the cell but also can be applied to localization of epitopes with potential in biorecognition (Manning *et al*. 2004). Based on the report of Accogli *et al*. (2017), the lectin histochemical technique is very useful in detecting the level of spermatozoa fertility based on the glycosylation process that occurs on the surface of spermatozoa. In addition, it was also found that there were extensive modifications in the formation of spermatozoa which were expressed by differences in the binding pattern between glycoconjugates and lectins.

Several studies on distribution of glycoconjugates in the epididymis using lectin histochemical methods have been reported, such as in the boar epididymal tissue (Calvo *et al*. 2000), canine (Schick *et al*. 2009), and alpaca (Parillo *et al.* 2009). However, studies to obtain data on the character, distribution and binding pattern between glycoconjugates and lectins in the epididymal tissue of Javan muntjac is not available. Therefore, the objective of this study was to determine the characteristic of glycoconjugates distribution pattern in three parts of epididymis namely caput, corpus, and cauda epididymis of Javan muntjac using lectin histochemical method.

MATERIALS AND METHODS

Animal and Tissue Sample

This study used two pair of epididymis obtained from two adult Javan muntjacs with hard antlers, aged two to four years, body weight 19-20 kg, and in healthy condition clinically. Male muntjacs have undergone several antler cycles namely hard antlers, casting, and velvet antlers. Muntjacs were obtained from Cilacap Regency, Central Java, with a capture permit based on SK. 23/Menhut-II/2011.

Histological Preparation of Epididymis

Epididymis organs were separated from the testes and fixed immediately in Bouin's solution for 24 hours and transferred to 70% ethanol. Afterwards, each epididymis was divided into three parts, namely caput, corpus, and cauda epididymis, each cut with a size of 0,5 cm and then dehydrated in graded series of ethanol (70%, 80%, 90%, and absolute), cleared in xylene, and embedded in paraffin. After dehydration process, samples were immersed in xylene solution, infiltrated in liquid paraffin, and embedded in paraffin. The paraffinized tissue was cut serially at 3 µm-thick sections using a manual microtome (Leica RM2235, Leica Biosystem, Nusslogh GmbH, Germany) and placed on slide. Furthermore, slides were stained with lectins histochemistry for detection of glycoconjugates distribution in caput, corpus, and cauda epididymis of muntjacs.

Lectin Histochemistry Staining

Prior to lectins histochemistry staining, all slides were dewaxed with xylene, and rehydrated with ethanol. In this present study, six biotinylated lectins: Peanut agglutinin (PNA), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA), Ricinus communis agglutinin (RCA), Concanavalin A (Con A), and Ulex europaeus agglutinin I (UEA I) (BK 1000, Vector Lab. Inc. Burlingame, USA) were used. Slides were then treated with Phosphate-buffered saline (PBS) pH 7.4, and incubated with 0.3% H_2O_2 in 0.01 M PBS pH 7.4 for 10 minutes at room temperature, followed by incubation with 2% normal goat serum for 30 minutes. The next steps were incubation slides with biotinylated lectins diluted with 0.01 M PBS (pH 7.4) at moist chamber overnight at 4° C, while the negative control slide was only dripped with PBS. The concentration of lectins used was 20 g/mL for PNA and 15 g/mL for other lectins (Table 1). After lectins incubation, slides were then rinsed with PBS and the incubated with the avidin biotin complex peroxidase (ABC) kit (Vectastain Elite ABC Kit Vector Lab. Inc. Burlingame, USA). Visualization of staining results was carried out by incubating slides with 3.3' diaminobenzidine which had been mixed with 0.05 M tris buffer HCl and 0.003% H_2O_2 for four minutes.

Table 1. The types of lectins, corresponding carbohydrates or sugar residue, and the optimal concentration of lectins used

Lectins acronym	Source	Specificity	Concentration (µg/mL)
PNA	*Arachis hypogaea*	B-D-Gal-(1,4)-D-GaNAc	20
SBA	*Glycine max*	a-D-GalNAc>B-D-GalNAc	15
Con A	*Canavalia ensiformis*	a-D-Man,>a-D-Glc	15
RCA	*Ricinus communis*	B-D-Gal-(1,3)-D-GaNAc	15
WGA	*Triticum vulgaris*	GlcNAc,>Sialic acid	15
UEA I	*Ulex europeus*	a-L-Fuc	15

PNA= Peanut agglutinin; SBA= Soybean agglutinin; Con A= Concanavalin A; RCA= Ricinus communis agglutinin; WGA= Wheat germ agglutinin; UEA 1= Ulex europaeus agglutinin I; GalNAc= N-acetylgalactosamine; Gal= Galactose; Man= Mannose; Glc= Glucose; GlcNAc= N-acetylglucosamine; Fuc= Fucose

Slides were then counterstained with Meyer's hematoxylin, subsequently dehydrated, cleared, and mounted using Entellan® (Merck, Germany).

Staining results were observed using a CH30 light microscope (Olympus, Japan) and photographed by a microscope equipped with a DP12 digital cammera (Olympus, Japan). The positive reaction of presence of glycoconjugates bonds with lectins on the epididymal tissue and the surface of spermatozoa was visualized by the formation of a brown color. Furthermore, the qualitative interpretation of the intensity was determined based on the following criteria, namely (-) negative staining, (±) weak, (+) moderate, (++) strong, and (+++) very strong intensity.

RESULTS AND DISCUSSION

The present study was the first to detect and characterize various pattern of glycoconjugates distribution in the epididymis of the Javan muntjac using lectin histochemical method. The data obtained from this study are required to complete data on glycoconjugates distribution during spermatogenesis that occurs in the seminiferous tubules of testicular tissue of the Javan muntjac as reported by Wahyuni *et al*. (2016). The epididymis shows a different structure in the three regions, which is characterized by the epididymal cell layer, the thickness of the muscle layer, and the number of spermatozoa present in the lumen of epididymal ductus. Additionally, several cell types lining the mucosal of ductus in epididymal muntjac have been identified, namely principal cells, basal cells, clear cells, and halo cells (Wahyuni *et al*. 2012a). In this study, the focus of observation on epididymal parts of caput, corpus, and cauda were principal cells, basal cells, spermatozoa, stereocilia, and stroma.

The distribution pattern of lectin binding glycoconjugates intensity that observed in caput, corpus, and cauda epididymis is presented in Table 2, Figure 1, and Figure 2. According to the binding affinity between the PNA, SBA, WGA, Con A, RCA, and UEA 1 lectins, it was found that there were differences in the distribution pattern as observed in caput, corpus, and cauda epididymis of Javan muntjac. A moderate to strong positive reaction has been found at the principal cells in the lamina epithelium of caput, corpus, and also cauda epididymis with PNA, SBA, WGA, Con A, and RCA lectins. However, negative result of UEA 1 lectin was found in almost component of epididymal tissue except in the stroma with weak intensity.

The presence of glycoconjugates with N-acetyl-galactosamine sugar residues in the principal cells, basal cells, and spermatozoa in the lumen of the caput were intensely detected by the PNA and SBA lectins. However, the distribution pattern found in the caput was different with the corpus and cauda epididymis with more even distribution throughout the epididymal epithelial layer, and progressively weaker in the corpus and then in cauda regions. Furthermore, glycoconjugates with sugar residues of N-acetyl-glucosamine and sialic acid that binding with WGA lectin were distributed in the caput and corpus epididymal epithelium with strong intensity of brown color found in the basal part of the caput epididymis, but the intensity tends to decrease in the epithelial layer of corpus epididymis. In addition to the epithelial layer, stereocilia and spermatozoa in the caput were also strongly positive with WGA lectin, but the intensity was slightly decreased in the corpus as well as in cauda epididymis.

The next lectin was Con A that binding with β-glucose and mannose sugar residues with strong intensity as found in the caput and corpus, while in the caudal part, the intensity resulted was moderate. Furthermore, the RCA lectins that bind to β-galactose and N-acetyl-galactosamine sugar residues were also found with moderate to strong intensity in caput,

Table 2. Lectin binding pattern in the caput, corpus, and cauda epididymis of the Javan muntjac

Epididymis	Lectins					
	PNA	SBA	WGA	Con A	RCA	UEA
Caput Epididymis						
Principal cells	++	+	++	++	+	-
Basal cells	+++	+++	+	+	+	-
Stereocilia	++	-	+	++	++	-
Spermatozoa	+	+	++	+	+	-
Stroma	++	±	++	++	++	-
Corpus Epididymis						
Principal cells	++	++	++	++	+	-
Basal cells	+++	++	++	+	+	-
Spermatozoa	+	++	++	++	+	-
Stereocilia	+	+	+	+	±	-
Stroma	++	+	++	++	++	-
Cauda Epididymis						
Principal cells	+++	+++	++	++	+	-
Basal cells	++	++	+	+	+	-
Stereocilia	+++	++	+	+	±	-
Spermatozoa	-	++	+	+	±	-
Stroma	++	+	++	+	+	-

PNA= Peanut agglutinin; SBA= Soybean agglutinin; Con A= Concanavalin A; RCA= Ricinus communis agglutinin; WGA= Wheat germ agglutinin; UEA 1= Ulex europaeus agglutinin I; GalNAc= N-acetylgalactosamine; Gal= Galactose; Man= Mannose; Glc= Glucose; GlcNAc= N-acetylglucosamine; Fuc= Fucose; -= Negative staining; ±= Weak; += Moderate; ++; Strong; +++= Very strong

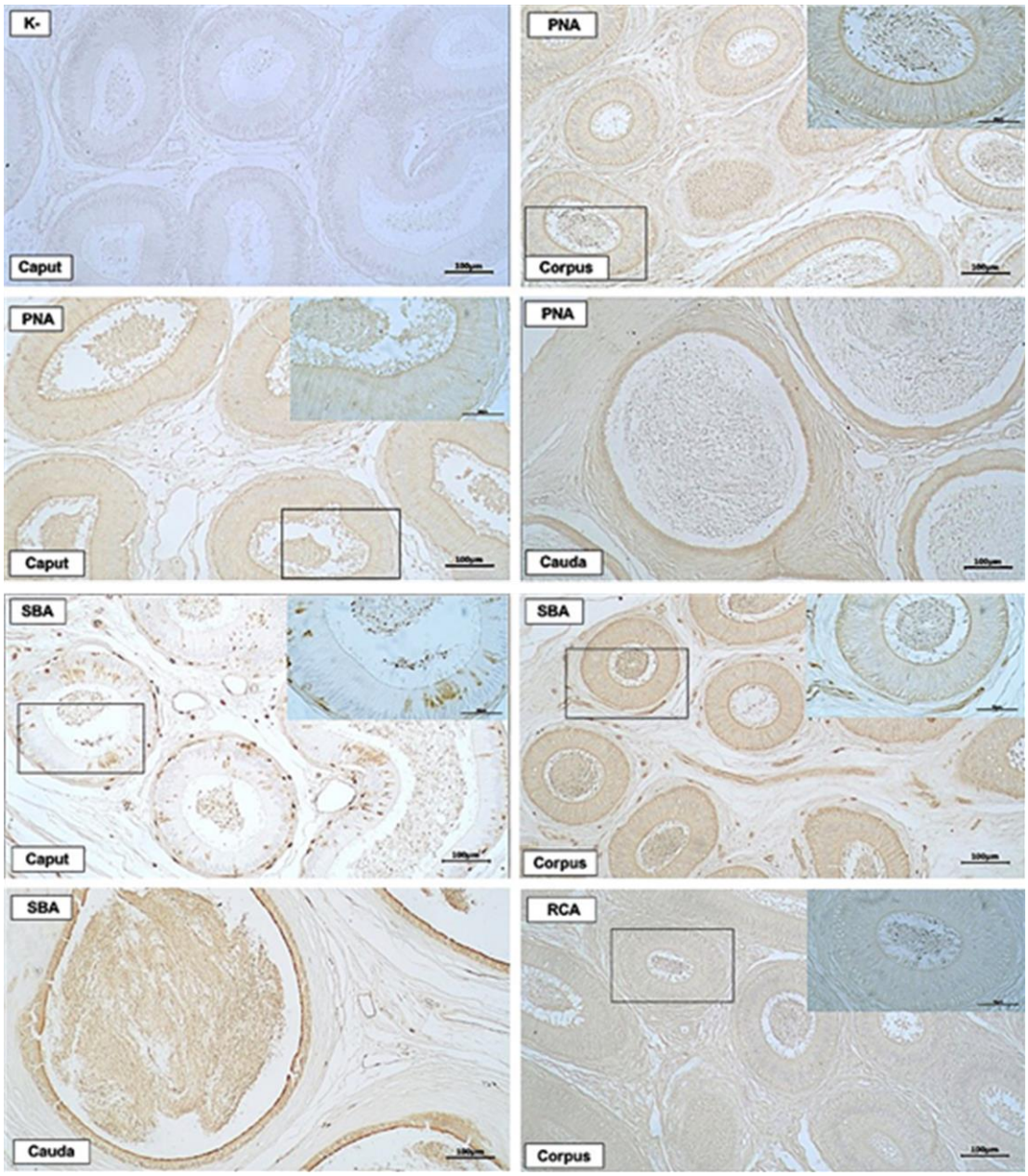

Figure 1. Distribution of glycoconjugates in the three epididymal regions of Javan muntjac. K-= Negative control; PNA= Peanut agglutinin; SBA= Soybean agglutinin; RCA= Ricinus communis agglutinin; N-acetyl-galactosamine sugar residues detected by PNA and SBA lectins; N-acetyl-galactosamine and galactose detected by RCA lectins. Lectin histochemical staining. Bar scale= 100 µm and 50 µm (inset)

corpus, and cauda epididymis. Although most of lectins reacted with strong and moderate intensity, only UEA 1 lectin binding fucose was negative in principal and basal cells, spermatozoa, stereocilia, and stroma of epididymis. This indicates the absence of fucose sugar residue in the three parts of epididymis of the Javan muntjac.

The presence of glycoconjugates that have been successfully localized in the principal cell (apical part and cytoplasm), stereocilia, and basal cells indicates that these cells play a role in synthesizing glycoproteins required in the process of sperm maturation (Hinton *et al*. 1996). Apical and stereocilia in the caput of epididymis were strongly positive reaction for PNA lectin. This indicates the importance of glycoproteins during the sperm maturation and transportation along the lumen of caput and corpus regions. Similar pattern of glycoprotein distribution has also been reported in the epididymis of humans (Arenas *et al*. 1998), cattle (Arya and Vanha-Perttula, 1985; Arya and Vanha-Perttula, 1986), boar (Calvo *et al*. 2000), and marsupial (Cooper *et al*. 2001).

Lectin histochemical methods have been widely applied to various animal species to detect the glycoconjugates distribution in the epididymis using several types of lectins (PNA, RCA, SBA, WGA, Con A, and UEA). In this present study, the SBA lectin has been showed the important role of glycoconjugates with sugar residue N-acetyl-galactosamine produced in the apical of principal cells. These glycoconjugates are needed to sperm maturation and maintaining the epididymal cells, especially the principal cells. N-acetyl-galactosamine were detected in large quantities mixed with spermatozoa in the lumen part of the caput

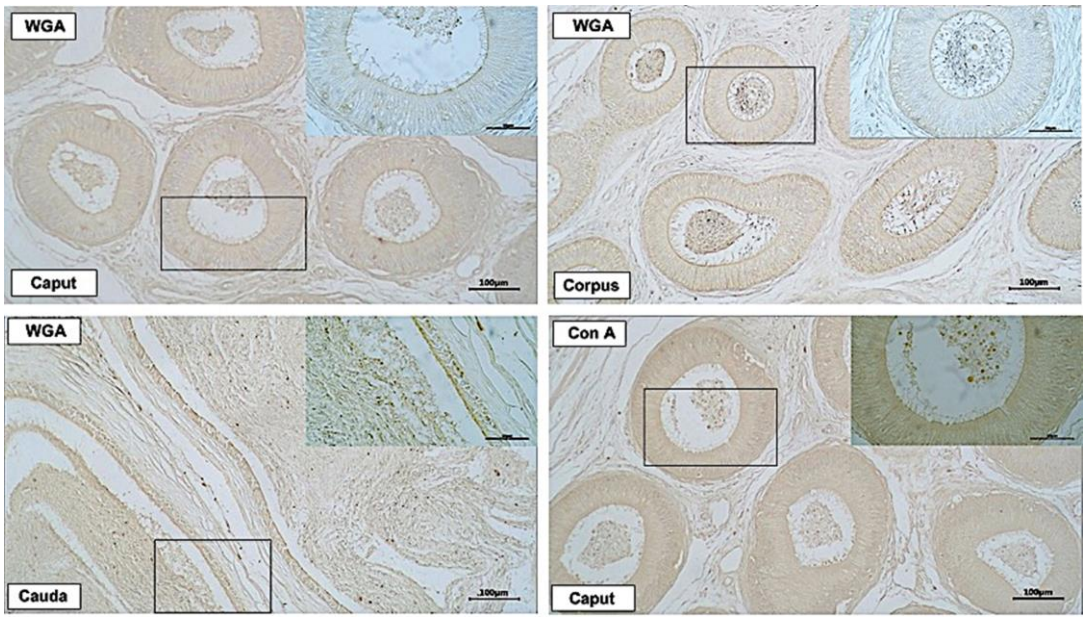

Figure 2. Distribution of glycoconjugates in the three epididymal regions of Javan muntjac. Con A= Concanavalin A; WGA= Wheat germ agglutinin. Positive WGA lectin in all three zones indicated N-acetyl-glucosamine and sialic acid; and Con A lectin detected mannose and glucose sugar residues. Lectin histochemical staining. Bar scale= 100 µm and 50 µm (inset)

epididymis. According to Eddy and O'Brien (1994), luminal glycoproteins produced by the epididymal epithelium will bind to the surface of spermatozoa during their transit in the lumen of epididymis. The results of this present study showed that glycoconjugates was more concentrated in the tail of spermatozoa which was strongly positive with WGA lectins. This pattern distribution was found throughout the epididymal regions through which spermatozoa passed and indicated the important role of glycoconjugates with sugar residues N-acetyl-glucosamine and sialic acid as reported in the epididymal tissue as well as on the sperm surface along the canine epididymis (Schick *et al*. 2009).

Another lectin that was also positive for spermatozoa was PNA lectin. These glycoconjugates with galactose and N-acetyl-galactosamine were detected strongly intensity from the caput to the corpus, but weak intensity was observed in the cauda epididymis of muntjac. These findings also reported on the surface of epididymal spermatozoa, particularly in the caput and cauda epididymis of marsupial that detected by using SBA and Con A lectins. Furthermore, spermatozoa in the cauda epididymis of marsupial also positive by WGA lectin indicate that terminal saccharides of sialic acid were present on the surface of spermatozoa (Cooper *et al*. 2001). The presence of sialic acid on the sperm surface of lesser mouse deer ejaculate still observed and evidenced by the positive reaction of WGA lectin (Prasetyaningtyas *et al*. 2006).

The presences of β-galactose and N-acetyl-galactosamine in spermatozoa were found only in the lumen of caput, and were negative in the lumen of corpus and cauda epididymis. Additionally, the absence of UEA in mostly parts of epididymal tissue in this Javan muntjac indicates that fucose sugar residue may not be present and also on the surface of spermatozoa transit along epididymal ductus. This result also reported in epididymal spermatozoa of marsupial marked by negative staining with FITC-UEA1 (Cooper *et al*. 2001). However, fucose sugar residue was observed in the principal cells of canine epididymis (Schick *et al*. 2009).

The occurrence of changes in the distribution pattern of glycoconjugates in glycoprotein-bound spermatozoa along the lumen of epididymal ductus indicate that the maturation process of spermatozoa occurring in both caput and corpus of epididymis. A change pattern was also observed in the spermatozoa located in the lumen of cauda with weak intensity of color than in proximal parts of epididymal ductus. This indicates that in the cauda, spermatozoa are mature and subsequently stored in this part before being ejaculated through the ductus deferens. The persistence of the positive reaction of WGA lectins in the cauda epididymis of Javan muntjac and other species is thought to be related to the protective function of glycoproteins with sialic acid sugar residues against mature spermatozoa. As terminal sugar of sialo-glycoproteins, sialic acid is synthesized and secreted by epididymal epithelium and then binds to the surface of spermatozoa during transit along epididymal ductus (Lassalle and Testart 1994). Some of the roles of sialic acid are protecting spermatozoa from antigens (Toshimori *et al*. 1988), supporting sperm binding to the epithelial cells in the oviduct reservoir zone (DeMott *et al*. 1995), and increased the spermatozoa binding ability to the zona pellucida of oocytes (Peterson *et al*. 1986).

In this present study, distribution of glycoproteins in the tissue of caput, corpus, and cauda epididymis of Javan muntjac can be detected using six types of lectins that have certain specificity for sugar residues of glycoconjugates. Therefore, the histochemical lectin method is very applicable to study the physiology of sperm maturation and storage in the epididymis of other subspecies muntjac as well as in other male species.

CONCLUSION

Glycoconjugates with sugar residues of β-galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, mannose, and β-glucose are distributed with different pattern in caput, corpus, and cauda epididymis that seem related to the specific function of each part of epididymis. The presences of sialic acid on sperm surface along the lumen of epididymis prove the role of sialic acid as a spermatozoa protective glycoprotein.

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