Determination of some of the lectins' immunohistochemistry profile of the dog testis and epididymis

Abstract: Lectins are substances composed of proteins or glycoproteins that specifically bond to carbohydrates on the cell surfaces or inside the cell. Lectins are used to identify the carbohydrate types in cellular membrane or cytoplasm. The aim of the study was to identify the location and distribution of the four types of lectins PNA (Peanut agglutinin- Arachis hypogea), WGA (Wheatgerm Agglutinin- Triticum vulgaris), Con A (Conconavalin A-Canavalia ensiformis) and SBA (Soybean Agglutinin-Glycine max) in dog testes and epididymites. The testes and the body of 10 adult dogs were collected from an animal shelter after castration. Following the standard histological procedures, lectin histochemistry was conducted on paraffin sections. In epididymal ducts and seminiferous tubules, the reaction was positive for PNA, WGA and SBA. The deferent duct reaction was positive in all types of lectins. Based on the study data, we can suggest that the analysis of the results could be beneficial for cell and tissue culture techniques, as well as the stem cell research, sperm maturation, canine capacitation and decapacitation.

Keywords: Dog, deferent duct, epididymis, testis, lectin histochemistry.

1. Introduction

Lectins were discovered more than 100 years ago and described by their ability to selectively recognize specific carbohydrate structures. They have been widely employed in histochemical studies to map glycosylation in cells and tissues [1].

The pathway where the primary spermatocyte is transformed into a sperm is a complex process and all changes in spermatogenic cells have not yet been clarified. An essential step in mammalian fertilization is the recognition and binding between spermatozoa and the egg's extracellular coat, the *zona pellucida* (ZP) [2]. Various carbohydrates such as galactose in α-linkage and N-acetylglucosamine in β- linkage were suggested as the complementary sperm receptors that mediate the primary bond between the spermatozoon and the ZP [3]. Previous studies in the literature revealed that the mammalian sperm plasma membrane surface is coated with various glycoproteins but the modification of only a few are known during sperm transit [4, 5]. Certain glycoproteins were suspected to act as decapacitation factors on the surface of epididymal sperm [6, 7], while others were accepted as capacitation factors [6, 8]. Furthermore, for reproduction, it was well documented that carbohydrate moieties on the sperm surface also play a key role in immune infertility [9, 10]. Most authors agreed that the complementary molecules present on the surface of the opposite gametes are involved in the sperm-egg interaction [2].

Based on the above-mentioned data, the denomination of the spermatocyte carbohydrate types in any species would lead to new insight in sperm-egg binding and immune infertility. Certain research evidenced dog spermatozoa-epididymal spermatozoa [11, 12], testicular, epididymal, vas and ejaculated spermatozoon [13], acrosome reactions [14]; however, there are no studies that focused on the internal male genital

organ tissues of dogs. In contrast, the paraffin sections were employed to visualize glycoprotein properties of the internal male genital organs and the sperm in the present study. That was why the authors decide to determine whether the four types of specific carbohydrates are bound to or not from dog sperm surface with the lectin histochemistry method.

The aim of the study was to demonstrate the carbohydrate profile of the dog testis and epididymis using paraffin sections and lectin histochemistry.

2. Material and Method

Testes and epididymites of ten adult dogs in 8 breeds (West Highland Terrier, German shepherd, Golden Retriever, Husky, Kangal, Pitbull, Dobermann, Boxer, Pointer) and mixed-breeds, all older than 2 years, and with normal body mass, and a body condition score of 4-6 were collected from the dogs in an animal shelter after the surgical castration operation performed with global standards. The ethics commitee approval was obtained from Hatay Mustafa Kemal University Ethics Committee (Decision no: 2016/7-4). The testis tissues were isolated, fixed in the formaldehyde buffer solution, and then embedded in paraffin blocks. The 5 µm sections were transferred to adhesive slides. After deparaffinization, the slides were treated with the hydrogen peroxide solution (%3) to prevent endogenous peroxidase activity. Then the slides were washed with PBS (pH:7.2-7.4) for rehydration. They were then incubated in the serum blocking solution (TA-125-UB, Thermo Fisher®). The employed lectins were selected based on zona pellucida carbohydrate profile to achieve a match between the sperm and its all previous forms and to understand the zona pellucida better. All lectins were diluted with PBS to 1:50 ratio and incubated on the slides with the tissues for 60 minutes at room temperature. The slides that were incubated with biotin labeled lectins were treated with biotin labeled secondary

antibodies. The slides were incubated with enzyme conjugate. Then, the slides were stained with AEC Chromogen Kit (SigmaAldrich®; AEC101) and counter-stained with Mayer's hematoxylin for 10 seconds and closed off in a water-based medium. For peroxidase labeled SBA, the slides were washed with PBS for 60-minute lectin application and treated with AEC Chromogen Kit (SigmaAldrich® AEC101). Then, the same procedure was repeated and the samples were examined under a light microscope.

The dog parotid gland sections were employed as the positive control. In the current study, three biotin labelled and one peroxidase labelled lectins were used. Details of the employed lectins are presented in Table 1.

3. Results

It was determined that Leydig cells exhibited a positive reaction with Con A (Fig. 1A, 1B); however, seminiferous tubules were negative except the germ cells (Fig. 1B). Secondary spermatocytes, which began to form an anterior head (acrosomal cap), exhibited a positive reaction with SBA, while Leydig cells did not, and the spermatids had a mild positive reaction (Fig 1C, 1D). For PNA, anterior head of the secondary spermatocytes and spermatids exhibited positive reactions. The basement membrane surrounding the seminiferous tubules was also positive; however, the Leydig cells were not, although the wall of the capillaries located between the Leydig cells was positive (Fig 1E). Positivity was also identified inside the seminiferous tubules in certain areas between the germ cells and spermatids, and the capillary wall exhibited a positive reaction with WGA (Fig 1F).

There was a positive reaction between epididymal and PNA in stereocilia and spermium (Fig. 2A). There was a mild positive reaction between SBA and the duct, but

the reaction with the spermium was positive. On the stereocilia line, there were a few positive areas (Fig. 2B, 2C). There was a mild positive reaction between the Con A and the epididymal duct in cell cytoplasm and the stereocilia, but the reaction with the spermium was positive (Fig. 2D). The stereocilia located at the epididymal duct lumen were strongly positive for WGA, while there were weak intracytoplasmic positive local areas, and the spermium was negative (Fig. 2E).

The deferent duct reacted positive on the stereocilia line to all lectin types (Fig. 2F, 2G, 2H,2I). PNA and WGA also reacted positive in the apical ends of the cells (Fig. 2H, 2I). Con A reacted positive with the apical cytoplasm of most deferent duct cells, while the reaction was positive only with the whole cytoplasm of certain cells (Fig. 2F). SBA reaction was positive only in certain sections. The findings on lectin staining of the dog testis and epididymis are presented in Table 2 and 3.

4. Discussion and Conclusion

The present study allowed us to identify the location and distribution of four types of lectins (PNA, SBA, Con A and WGA) in the testes and epididymites of ten dogs. It is known that there are no seminal vesicles in dogs; thus the dog sperm should mature without the mediation of seminal vesicle secretions or fructose [15]. And maturation of the spermatogenic cells requires new glycoproteins and carbohydrates [16]. Thus, the study was conducted on dog testis and epididymis. The seminal vesicles secrete fructose which is the main source of spermatozoan energy, and protects spermatozoa against reactive oxygen species (ROS) via the antioxidants [17]. After ejaculation, seminal plasma isolates the antibacterial acidic field in the vagina (pH 4–4.5), inhibits the immune reaction, and transports spermatozoa to the cervix. It contains factors that disrupt the capacitation of spermatozoa to prevent early activation and plays a role in the

implantation of the fertilized ovum with progesterone. The seminal fluid also assists the sperm-oocyte interaction by preserving the molecular structure of the spermatozoa [18]. The absence of all the above-mentioned functions of the seminal vesicles in dogs may lead to the coverage of the spermatozoa with other carbohydrate or glycoprotein types, in contrast to other species.

During epididymal transit, spermatozoa are mixed with the epididymal secretory fluid [16]. It is clear that the secretory material attaches probity to the sperm surface, modifying the lectin staining pattern. These secretory products also include several enzymes such as glycosidases and glycosyl-transferases, which could alter the terminal sugar residues on the sperm plasma membrane. Thus, it is important to examine both the tissues and the sperm or the ejaculated sperm.

Certain studies conducted lectin histochemistry in dogs [11-14], however, it should be emphasized that these studies basically focused on the ejaculated sperm instead of the tissues. The present study aimed to determine how the new sugar residues are attached to the carbohydrate chains, and/or the initial chains undergo further processing during glycoprotein synthesis in dog testis and epididymis.

Toyonaga et al. [19] reported that all spermatozoa obtained from different parts of the feline testis exhibited positive reactions with FITC-Con A, FITC-WGA and FITC-PNA. In our study, we found that WGA was negative for spermium. The present study findings also indicated that no β- linked N- acetylglucosamine and sialic acid residues existed in the dog spermium based on the histochemical analysis conducted on the paraffin sections. Also, none of the findings reported in previous studies was consistent with our results for the former spermium forms. This demonstrated that the lectin binding affinity of sperm glycoproteins alters as the sperm matures, and supported the concept

that sperm cells undergo a series of biochemical and physiological changes that require incorporation of new testicular and epididymal molecules [20].

In a study by Desantis et al. [21] conducted on cats, it was reported that none of the Leydig cells, Sertoli cells and spermatogonia were stained with PNA. However, they also reported that spermatocytes were faintly visible and the spermatids strongly reacted with PNA, consistent with our results. Thus, it could be suggested that β- linked galactose residues on spermatid is the same in both dogs and cats. In the same study conducted by Desantis et al. [21], SBA reacted mild positive with spermatids, and did not react with Leydig cells, Sertoli cells, spermatogonia and spermatocytes, and these findings were not consistent with our results except for the Leydig cells. Thus, for α and β linked N-acetylglucosamine, dog and feline glycoprotein profiles are different in the seminiferous tubules. Desantis et al. [21] determined that Con A reacted positively with Leydig cells, Sertoli cells, spermatogonia, spermatocytes and spermatids in various strengths. In our study, Con A only reacted positively with Leydig cells and this was not consistent with the findings reported by Desantis et al [21]. These results also demonstrated that α-linked mannose, α- linked glucose and N-acetylglucosamine properties of the feline and dog sperms are different. In studies conducted with dogs, these findings were possibly due to the initiation of protein glycosylation by the attachment of mannose and glucose sugar chains that react with Con-A. Neither cats nor dogs have seminal vesicles. However, dogs have ampullar glands. Our results indicated differences which could be due to the presence of ampullary gland secretions that cover the sperms. During maturation and fertilization of the spermatids, all secretions play crucial roles and each part of the internal organ has the potential to effect the other.

Maekawa and Nishimune [22] reported that the lectin PNA could be employed to separate the somatic and germ cells in mouse testis due to the germ cell affinity. Our results also demonstrated that the PNA lectin could also serve as a marker for dog testis germ cells.

The present study was conducted to determine the dog testis and epididymis carbohydrate content and to detail the sperm maturation process in mammals without a seminal vesicle. As the best to the authors' knowledge, the present study was the first to investigate carbohydrate profile of testis and epididymis tissues and the previous forms of the sperm in dogs. Based on the present study findings, it could be suggested that not only sperm, but also the testicular and epididymal tissues and secretions of the accessory glands specific to each species leads to specific egg-sperm binding mechanisms. The whole process should be investigated to scrutinize the maturation of the spermatocyte and the authors are in the process of planning further studies where the accessory glands will be investigated. The present study findings also suggested that lectins might be employed as diagnostic markers in dog infertility problems and could provide a stepping stone for further studies in the field.

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Ethical Statement: This study was approved by the Hatay Mustafa Kemal University Ethics Committee (Decision no: 2016/7-4)

Conflict of Interest

The authors declared that there is no conflict of interest.

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Table 1: Details of Lectins Used

Name of the lectin	Nominal Sugar Specifity	Dilution	Source
PNA	α- linked galactose	1:50	SigmaAldrich®
SBA	α and β linked N-accetylgalactoseamine	1:50	SigmaAldrich®
WGA	β linked N-acetylglucosamine sialic acid	1:50	SigmaAldrich®
Con A	α- linked mannose α- linked glucose α- linked N-acetylglucosamine	1:50	SigmaAldrich®

Table 2: PNA, SBA, Con A, WGA binding in testis and epididymis

Lectin	Leydig Cells	Basement Membrane	Sertoli Cells	Deferent Duct	Epididymal Duct
PNA	-	+	-	+	+
SBA	-	-	-	+/-	+/-
Con A	+ (c)	-	-	+	+
WGA	-	-	-	+/-	++

(-):no staining (+): staining (+/-): faintly visible or light staining (+ +): strong staining (c): cytoplasmic staining

Table 3: PNA, SBA, Con A, WGA binding of spermatogenic cells

Lectin	Spermatogonia	Spermatocytes	Spermatids	Spermium
PNA	-	+ (a)	+	+
SBA	-	+ (a)	+/-	+
Con A	-	-	+/-	+
WGA	-	-	+	-

(-):no staining (+): staining (+/-): faintly visible or light staining (+ +): strong staining (c): cytoplasmic staining (a): apical zone

Figure Legends

Figure 1: Con A, SBA, PNA, WGA lectin binding in the dog testis. (**Fig 1A:** Con A binding, asteriks: Leydig cells, **Fig 1B:** Con A binding, asteriks: Leydig cells, **Fig 1C:** SBA binding, the seminiferous tubules, **Fig 1D:** SBA binding, the seminiferous tubules, **Fig 1E:** PNA binding, the seminiferous tubules, **Fig 1F:** WGA binding, the seminiferous tubules)

Figure 2: Con A, SBA, PNA, WGA lectin binding in the dog epididymis. (**Fig 2A:** PNA binding, epididymal duct, the corpus, **Fig 2B:** SBA binding, epididymal duct, the corpus, **Fig 2C:** SBA binding, epididymal duct, the corpus arrow head: positive reaction, **Fig 2D:** Con A binding, epididymal duct, the corpus, **Fig 2E**: WGA binding, epididymal duct, the corpus, **Fig 2F:** Con A binding, the deferent duct, **Fig 2G:** SBA binding, the deferent duct, **Fig 2H:** PNA binding, the deferent duct, **Fig 2I:** WGA binding, the deferent duct)

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