Variations of Sugar Expressions in the Female Genital Mucosa

雌性生殖器粘膜における糖鎖発現の変化

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Contents

I. Preface

The mucosa of the female genitalia is always exposed to microorganisms from the external environment, because the female reproductive organ is a special part of mating, pregnancy and delivering. In addition, the genital tract is continuously opened to the outside adjacent to the anus. The mucosal immune system is well developed in the genital mucosa to prevent microbial infection, while tolerating the fetus which is nonself for the maternal immune system during pregnancy (Thaxton et al., 2010; Wira et al., 2005). The immunologic first defense line is an innate immune system, and activation of the innate immunity is critical as well as induction of the subsequent antigen-specific adaptive immune responses.

On the other hand, it has been reported that the endometritis or chorioamniotis and the pyometra have been considered to occur by bacterial infection from the vagina in mammals (Leitner et al., 2003). For example, canine pyometra is seemed to be clinically the most important pathologic condition in the uterus. It has been suggested that the basis of the disease is

endometritis with cystic glandular hyperplasia, and that superimposition of pathogenic organisms often results in the development of pyometra in bitches. There has been considerable debate over predisposing factors for this disease. The primary determinant is uterine dysfunction due to ovarian abnormality. Many workers have investigated changes in the concentration of ovarian steroid hormones in blood and changes in their receptors in uteri with pyometra. However, they failed to conclude that these were the only factors involved (De Bosschere et al., 2001). The bacterium most frequently isolated from the canine uterus is Escherichia coli. The pyometra is most commonly observed in the first half of diestrus; the period during which severe pyometra can be induced by inoculation of *E. coli* into the uterus was limited to the early stage of diestrus (Ishiguro et al., 2007 Kida et al., 2006). Conversely, it has showed in normal itches, that bacteria were always detected in the uterus during proestrus and estrus, but rarely at other stages of the estrous cycle (Ohlsen et al.; 2009). It has been believed that bacteria enter the uterus through the patent cervical canal during estrus. Although bacteria are present in the

lumen, pyometra rarely occurs during proestrus and estrus. The canine uterus might therefore have some protective capability from bacterial infection during these intervals, which is subsequently lost in the first half of diestrus. However, it has also been reported that the adhesion of microbes to the host mucosa is mediated by their own lectin-like molecules which combine specific glycoconjugates on the cell surface selectively (Hultgren et al., 1993; Mouricout and Vedrine., 2000). These facts indicate that bacterial infection of the endometrium would induce pyometra mediated by bacterial lectins (Leitner et al., 2003).

From these facts, I decided to investigate sugar expressions on mucosae of the uterus and vagina of the pregnant mice and bitches with pyometra, using a panel of lectins, in order to clarify the pathogenesis of the endometritis, chorioamniotis, pyometra and pregnancy failure.

In Chapter 1 of this thesis, expression patterns of sugars on the mucosal surface of the uterus in pregnant mice were investigated in both mucosal surfaces of the superficial epithelial cells of the uterus, the mesometrial side

where the placenta was developed; the anti-mesometrial side where the implantation took place.

In Chapter 2, sugar expressions were examined on the epithelium of both the middle portion of the vagina and the vaginal portion of the cervical canal in pregnant mice.

Finally in Chapter 3, sugar expressions on mucosae of the uterus and vagina were investigated with the normal estrous cycle, which were categorized as immature, estrus, days 7-10 of diestrus, days 30-40 of diestrus, young anestrus, aged anestrus, and those with the pyometra.

II. Chapter 1

Lectin Histochemistry for Sugars on the Mucosal Surface of the Uterus in Pregnant Mice

Abstract

Expression patterns of sugars on the mucosal surface of the uterus in pregnant mice were investigated by using 21 kinds of lectins. In the uterine mucosa, the GlcNAc group tended to express a positive reaction before pregnant day 10, but the glucose/mannose group generally expressed a positive reaction after pregnant day 10. On the other hand, the fucose group expressed a negative reaction during all periods in pregnancy. These findings were almost the same on both the mesometrial side and anti-mesometrial side of the uterus. These differences of sugar expression probably reflect the functional change of the mucosa during pregnancy and the alteration of sugar expression may give a chance for pathogens to infect in the uterus with limited periods.

Keywords: lectin, mouse, pregnant, sugar, uterus.

Introduction

The orifice of the female genital tract is constantly opening toward an external environment, and thus the mucosal surface of the genital tract is

habitually exposed to the risk of infection by microorganisms. To prevent the invasion of external pathogens, an innate immune system is developed in the mucosa of the uterus (Thaxton, et al., 2010; Wira et al., 2005). On the other hand, it has been reported that the human endometritis or chorioamniotis and the canine pyometra have been considered to occur by bacterial infection from the vagina (Hickey et al, 2011; Leitner et al., 2003; Spiegel, 1991).

In general, the adhesion of microbes to the host mucosa is mediated by their own lectin-like molecules which combine specific glycoconjugates on the cell surface selectively (Hultgren et al., 1993; Mouricout and Vedrine, 2000). In addition, the structures of genital tract epithelia are changed during the estrous cycle and pregnancy (Gonzalez et al., 2007). Therefore, the expression of sugars on the genital tract epithelia should be clarified to better understand the bacterial infection in the uterus. However, the sugar expression on the uterus has never been clarified (Horvat et al., 1993).

In this study, we surveyed the expression patterns of sugars on the mucosal surface of the uterus in pregnant mice by using 21 kinds of lectins. Lectins are

defined as glycoproteins that express specific binding activity to carbohydrates, and have been used as a selective marker to identify the specific sugar-expressed cells in mammals (Gabius, 2001; Yamamoto et al., 2010).

Materials and Methods

This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 10-T-47), and all procedures were conducted according to the Guide for the Care and Use of Laboratory Animals at Tottori University. Male and female BALB/c mice aged 8 weeks (CLEA Japan, Japan) were reared under conventional laboratory housing conditions that allowed us to obtain pregnant mice. The day when the plug was found was defined as the first day of pregnancy. Non-pregnant mice were also prepared as a control. Pregnant mice on pregnant day (P) 2, P7, P10, P13 and P18, and non-pregnant mice on diestrus were sacrificed (N=5, respectively) by *i.p.* administration of 200 mg/kg pentobarbital sodium (Dainippon Sumitomo Pharmaceuticals, Japan).

The uterus (middle portion between the cervix of the uterus and uterine ostium of tube) was dissected and immediately immersed in Bouin's solution for 16 hrs at room temperature. Tissue samples were embedded in paraffin according to a routine procedure, and sections of 4 μm thickness were pretreated with serum-free protein block (X0909; Dako, Denmark) for 1 hr at room temperature following elimination of the endogenous peroxidase activity with 0.03% H_2O_2 and 100% methanol for 30 min, respectively. Thereafter, the samples were incubated with 21 kinds of biotinylated lectins at a 1:5000 dilution (BK-1000, 2000, 3000; Vector Laboratories, U.S.A.) for 18 hr at 4°C and with peroxidase-labeled streptavidin (424011; Nichirei, Japan) for 30 min at room temperature. Finally, the samples were reacted with 3, 3-diaminobenzidine (K4007; Dako).

Results and Discussion

The major specific binding properties of lectins for sugars can be roughly classified into the following 6 groups: N-acetylgalactosamine (GalNAc) group,

N-acetylglucosamine (GlcNAc) group, galactose group, glucose/mannose group, oligosaccharide group and fucose group (Table 1). In the uterine mucosa the sugar expression was examined both on the mucosal surface of the superficial epithelial cells of the mesometrial side where the placenta would be developed, and on the luminal surface of the anti-mesometrial side where the implantation would occur.

In the GalNAc group, RCA_{120} expressed a positive reaction with sugars through the gestation period, but most of the lectins did not express positive reactions during any of the periods (Table 2). In the GlcNAc group, LEL and STL tended to express a positive reaction before P10, but WGA expressed a positive reaction during all periods (Table 2, Fig. 1). In the galactose group, only JCA expressed a positive reaction during all periods (Table 2). In the glucose/mannose group, LCA and PSA tended to express positive reactions after P10, but ConA expressed a positive reaction duirng all periods (Table 2, Fig. 2). In the oligosaccharide group, PHA-E expressed a positive reaction during almost all periods, but PHA-L expressed a negative reaction during all

periods (Table 2). In the fucose group, UEA-I expressed a negative reaction during all periods (Table 2). All these findings were almost the same both on the mesometrial side and the anti-mesometrial side (Table 2).

A mutual relation between the mucosa and microorganisms has been observed in many species. In the gastrointestinal tract, bacteria selectively adhere to certain sugars on the mucosa by using their own microbial lectins (Guarner and Malagelada, 2003; Hultgren et al., 1993; Mouricout and Vedrine, 2000). The sugar expressions differ among the segments of the alimentary tract, therefore the site differences of sugars are thought to reflect the different settlement of indigenous bacterial species (Takeuchi et al., 2007; Yamamoto et al., 2010). A large number of indigenous bacteria are also residing at the genital mucoGsa to constitute microflora (Noguch et al., 2003; Reid, 2001) and the mucosal immune system is organized in the genital tract to maintain these physiological conditions (Gonzalez et al., 2007; Soboll et al., 2006). In the present study, the GlcNAc group tended to express a positive reaction before

P10 and the glucose/mannose group generally expressed a positive reaction after P10 in the uterine mucosa.

It is suggested from these results that the change of the sugar expression on the mucosal surface would occur not only site-specifically but also time-dependently and the differences in sugar expressions probably reflect the functional change of the mucosa during pregnancy in the mouse uterus. On the other hand, the expression patterns of sugars on the uterine mucosa were almost the same both on the mesometrial side and anti-mesometrial side of the uterus, whereas each of mucosa has different function in pregnancy as placentation and implantation, respectively. It is known that glycoconjugates on the endometrium play an important role for recognition and implantation of the embryo in pregnancy (Leitner et al., 2003). Furthermore the number of *Escherichia coli* on the endometrium increased only at day 10 of dieestrus which correspond to the implantation period and adhesion of *E. coli* to endometrial epithelial cells was also inhibited by addition of D-mannose at day 10 of dieestrus in the canine uterus (Ishiguro et al., 2007).

These facts indicate that the endometrium does not express specific sugars constantly which allow some bacteria to bind selectively and these sugar expressions on the endometrium would alter in the short term. Taken together, it is speculated that the alteration of sugar expression on the mucosa may consequently give a chance for pathogens to attach to the mucosal surface to cause the infection in the uterus with limited periods. Further studies need to be investigated about an association between mucosal sugars and bacterial adherence in the genital tract.

Table 1. Lectins and sugar specificities

Lectin	Abbreviation	Major sugar specificity
N-acetylgalactosamine group		
Arachis hypogaea	PNA	β-D-Gal(1–3)D-GalNAc
Bandeiraea simplicifolia-I	BSL-I	α-D-Gal, α-GalNAc
Dolichos biflorus	DBA	α-D-GalNAc
Glycine maxi	SBA	α-D-GalNAc-3Gal
Ricinus communis	RCA_{120}	β-D-Gal-4GlcNAc, D-Gal and D-GalNAc
Sophora japonica	SJA	β-D-GalNAc
Vicia villosa agglutinin	VVA	D-GalNAc-3GalNAc
N-acetylglucosamine group		
Bandeiraea simplicifolia-II	BSL-II	D-GlcNAc
Datura stramonium	DSL	$(GlcNAc)_2$
Lycopersicon esculentum	LEL	$(GlcNAc)_{2-4}$
Solanum tuberosum	STL	$(GlcNAc)_{2-4}$
Succinylated WGA	s-WGA	$(GlcNAc)_n$
Triticum vulgaris	WGA	$(GlcNAc)_n$, NeuNAc
Galactose group		
Artocarpus integrifolia	JCA	β-D-galactopyranoside
Erythrina cristagalli	ECL	β-D-Gal(1–4)D-GlcNAc
Glucose/mannose group		
Canavalia ensiformis	Con A	α-D-Man, α-D-Glc
Lens culinaris	LCA	α-D-Man
Pisum sativum	PSA	α-D-Man
Oligosaccharide group		
Phaseolus vulgalis-erythroaggulutinin	PHA-E	bisected, triantennary N-glycans
Phaseolus vulgalis-leucoagglutinin	PHA-L	bisected, triantennary N-glycans
Fucose group		
Ulex europaeus-I	UEA-I	α-L-Fuc-α-D-Gal-β(1–4)GlcNAc

Fuc: fucose, Gal: galactose, GalNAc: N-acetylgalactosamine, Glc: glucose, GlcNAc: N-acetylglucosamine, Man: mannose, NeuNac: N-acetylneuraminic acid.

Table 2. Specific sugar expression on the mouse uterine mucosa

Lectin	D		P2		P7		P10		P13		P18	
	M	A	M	A	M	A	M	A	M	A	M	A
PNA	-	-	-	-	-	-	-	-	-	-	-	-
BSL-I	-	-	-	-	-	-	-	-	-	-	-	-
DBA	-	-	-	-	-	-	-	-	-	-	-	-
SBA	-	-	-	-	+	+	-	-	-	-	-	-
RCA_{120}	-	-	++	++	++	++	+	+	+	+	++	++
SJA	-	-	-	-	-	-	-	-	-	-	-	-
VVA	-	-	-	-	-	-	-	-	-	-	-	-
BSL-II	-	-	-	-	-	-	-	-	-	-	-	-
DSL	-	-	-	-	-	-	-	-	-	-	-	-
LEL	+	+	++	++	++	++	+	+	-	-	-	+
STL	++	++	+	+	+	+	-	-	-	-	-	-
s-WGA	-	-	-	-	-	-	-	-	-	-	-	-
WGA	++	++	++	++	+	+	+	+	+	+	+	+
JCA	+	+	+	+	++	++	+	+	+	+	++	++
ECL	-	-	-	-	-	-	-	-	-	-	+	-
Con A	++	++	+	+	+	+	++	++	+	+	++	++
LCA	-	-	-	-	-	-	-	-	+	+	+	+
PSA	+	+	-	-	-	-	+	+	+	+	+	+
PHA-E	+	+	-	-	+	+	+	+	++	++	+	+
PHA-L	-	-	-	-	-	-	-	-	-	-	-	-
UEA-I	-	-	-	-	-	-	-	-	-	-	-	-

Fuc: fucose, Gal: galactose, GalNAc: N-acetylgalactosamine, Glc: glucose, GlcNAc: N-acetylglucosamine, Man: mannose, NeuNac: N-acetylneuraminic acid. D: diestrus, P: pregnant day, M: mesometrial side, A: anti-mesometrial side. ++: strong staining, +: moderate staining, -: negative staining.

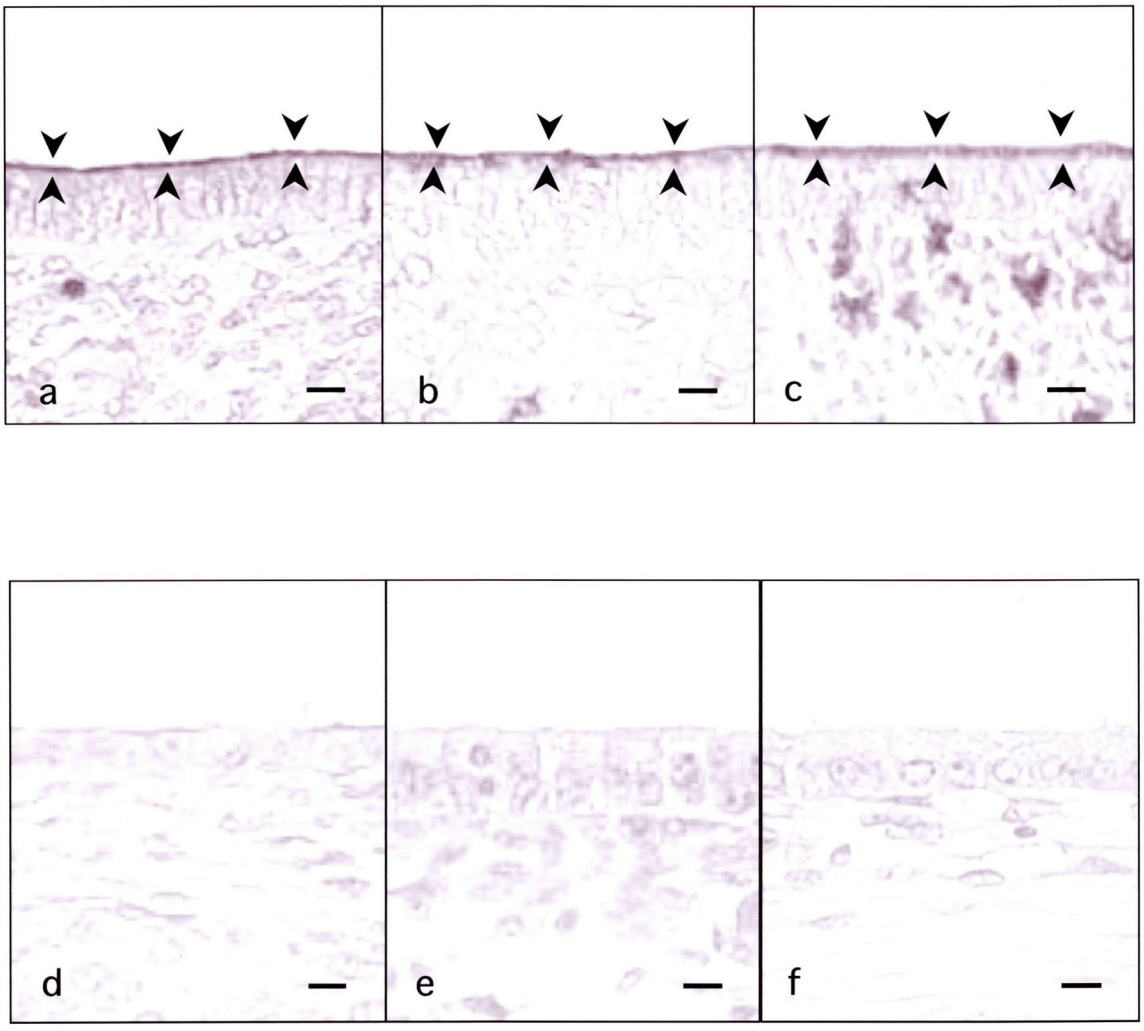

Fig. 1. Lectin staining patterns of STL on the uterine mucosa of the mesometrial side. Apical surfaces of the mucosa express a positive reaction (arrowheads) for STL on diestrus (a), pregnant day (P) 2 (b) and P7 (c), but a negative reaction on P10 (d), P13 (e) and P18 (f). Bar=10 μm.

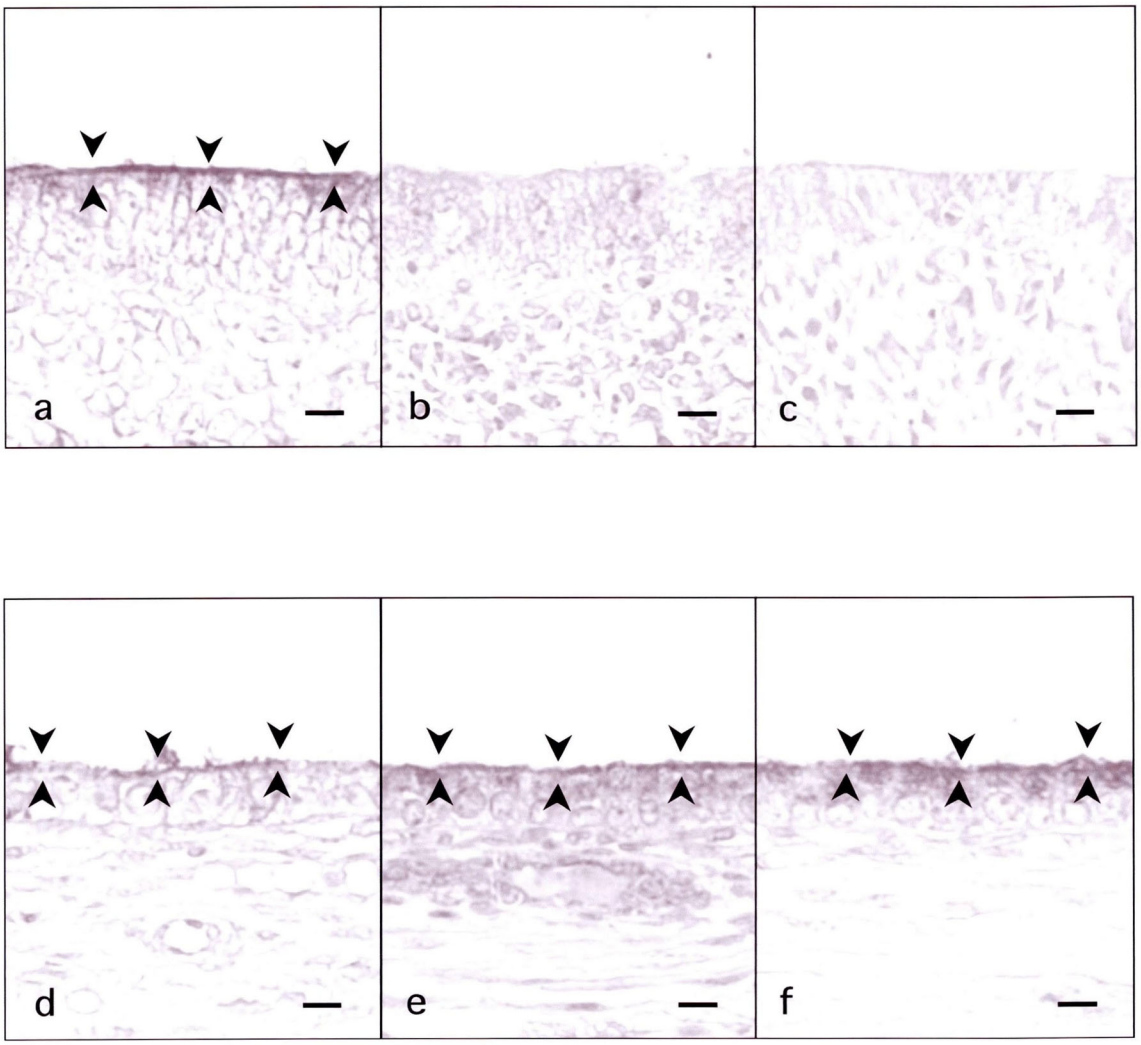

Fig. 2. Lectin staining patterns of PSA on the uterine mucosa of the mesometrial side. Apical surfaces of the mucosa express a negative reaction for PSA on pregnant day (P) 2 (b) and P7 (c), but a positive reaction (arrowheads) on diestrus (a), P10 (d), P13 (e) and P18 (f). Bar=10 μm.

III. Chapter 2

Sugar Expressions on the Vaginal Epithelium in Pregnant Mice

Abstract

Sugar expressions were examined on the epithelium of both the middle portion of the vagina and the vaginal portion of the cervical canal (CC) in pregnant mice to understand the pathogenesis of bacterial infection in the female reproductive organ by using a panel of lectins. As a result, N-acetylglucosamine was positive before pregnant day (P) 7, but negative after P10 and at diestrus on both the vagina and the CC. In addition, some differences in sugar expressions were seen between them. These results suggest that sugar expressions on the mucosal surface would change not only site-specifically but also time-dependently, and these sugar differences indicate the possibility of the alteration of the settled bacterial species on the vaginal mucosa in pregnancy.

Keywords: lectin, mouse, pregnancy, sugar, vagina.

Introduction

Infection by pathogens is one of the most important causes of infertility and abortion in the female reproductive organ (Wira et al., 2005). For instance,

human endometritis or chorioamnionitis and canine pyometra have thought to occur with bacterial infection via the vagina (Hickey et al., 2011; Leitner, et al., 2003; Spiegel, 1991). On the other hand, indigenous bacteria reside in the mucosa of the vagina and prevent the invasion of external pathogens in mammals (Noguchi et al., 2003; Reid, 2001).

The adhesion of microbes to the host mucosa is mediated by their own lectin-like molecules which selectively combine with specific glycoconjugates on the cell surface (Guarner and Malagelada, 2003; Hultgren et al., 1993; Mourincout and Vedrine, 2000). Clarification of sugar expressions on epithelia of the genital tract may help us to understand the pathogenesis of bacterial infection in the female reproductive organ, whereas there are only a few reports about sugar expressions on vaginal epithelia during the estrous cycle (Horvat et al., 1993) and sugar expressions have never been clarified on the vagina during the gestation period.

The aim of this study was to survey the expression pattern of sugars on the mucosal surface of the vagina and the vaginal portion of the cervical canal in

pregnant mice by using a panel of lectins. Lectins have been used as a selective marker to identify sugar-expressed cells to utilize their binding capabilities with specific sugars in mammals (Gabius, 2001; Yamamoto et al., 2010).

Materials and Methods

This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 10-T-47), and all procedures were conducted according to the Guide for the Care and Use of Laboratory Animals at Tottori University. Male and female BALB/c mice aged 8 weeks (CLEA Japan, Japan) were reared under conventional laboratory housing conditions that allowed us to obtain pregnant mice. The day when the vaginal plug was found was defined as the first day of pregnancy. Non-pregnant mice were also prepared as a control. Pregnant mice on pregnant day (P) 2, P7, P10, P13 and P18, and non-pregnant mice on diestrus (D) were sacrificed (N=5, respectively) by *i.p.* administration of 200 mg/kg pentobarbital sodium (Dainippon Sumitomo Pharmaceuticals, Japan).

The vagina (middle portion between the vaginal orifice and the cervix of the uterus) and the vaginal portion of the cervical canal (CC) were dissected and immediately immersed in Bouin's solution for 16 hr at room temperature. Tissue samples were embedded in paraffin according to a routine procedure, and sections of 4 μm thickness were pretreated with serum-free protein block (X0909; Dako, Denmark) for 1 hr at room temperature following elimination of the endogenous peroxidase activity with 0.03% H_2O_2 and 100% methanol for 30 min, respectively. Thereafter, samples were incubated with 21 kinds of biotinylated lectins at a 1:5000 dilution (BK-1000, 2000, 3000; Vector Laboratories, U.S.A.) for 18 hr at 4°C and with peroxidase-labeled streptavidin (424011; Nichirei, Japan) for 30 min at room temperature. Finally, samples were reacted with 3, 3-diaminobenzidine (K4007; Dako).

Results and Discussion

The major specific binding property of lectins for sugars can be generally classified into 6 groups as follows: N-acetylgalactosamine (GalNAc), galactose

(Gal), N-acetylglucosamine (GlcNAc), glucose or mannose (Glc/Man), fucose (Fuc) and oligosaccharide (OS) (Table 3).

In the GalNAc, all of lectins were negative through the gestation period and at D on mucosae of both the vagina and the CC (Table 4). In Gal, PNA were positive only at P2 on both the vagina and the CC, and JCA was positive through the gestation period and at D on both the vagina and the CC (Table 4). RCA_{120} was positive through the gestation period on the vagina, but it was negative at D on the vagina and at P10, P13 and D on the CC (Table 4, Fig. 3). In GlcNAc, DSL and STL were positive before P7, but they were negative at D on the vagina and at P10, P13, P18 and D on both the vagina and the CC (Table 4, Fig. 4). In addition, LEL was positive through the gestation period on the vagina, but it was negative at D on the vagina and at P10, P13, P18 and D on the CC (Table 4). WGA was positive through the gestation period and at D, but BSL-II was negative on both the vagina and the CC (Table 4). In Glc/Man, Con A was positive through the gestation period and at D except for P2 on both the vagina and the CC (Table 4). LCA and PSA were negative through the

gestation period and at D on both the vagina and the CC (Table 4). In Fuc, UEA-I was negative through the gestation period and at D on both the vagina and the CC (Table 4). In OS, PHA-E was positive at D and through the gestation period except for P2 on both the vagina and the CC, whereas PHA-L was negative through the gestation period and at D on both the vagina and the CC (Table 4).

Sugar residues on the apical cell membrane of the mucosal surface are utilized as binding targets for pathogens in infection, e.g. *Escherichia coli* is known to bind with GalNAc, GlcNAc and Man (Beachey 1981; Ohlsen et al., 2009). In the rabbit and rat alimentary tract, sugar expressions differ among segments of the tract and these variations are thought to reflect the different settlement of indigenous bacteria (Takeuchi et al., 2007; Yamamoto et al., 2010). A large number of bacteria also reside at the genital mucosa in many kinds of experimental animals (Noguchi et al., 2003; Reid, 2001) and sugar expressions such as GlcNAc and Glc/Man change during the gestation period on the uterine mucosa in pregnant mice (Yasunaga et al., 2012). In the present

study, GlcNAc were positive before P7 and negative after P10 on mucosae of both the vagina and the CC. By contrast, different lectin binding patterns were reported in many species during the estrous cycle (Leitner et al., 2003) which suggests the variety of sugar expressions among species. Further studies are needed to investigate an association between mucosal sugars and the bacterial adherence to elucidate the pathogenesis of bacterial infection in the female reproductive organ.

On the other hand, some differences were seen in sugar expressions between mucosae of the vagina and the CC. The CC corresponds to the transition site from the vagina to the uterus. The lumen of the cervical canal forms narrow-shape probably to prevent the invasion of external pathogens from the vagina to keep the uterus germ-free. However, bacteria were always seen in the uterine mucosa during proestrus and estrus stages in bitches while pyometra seldom occurred during these stages (Kida et al., 2006). Moreover, the number of *E. coli* on the endometrium increased only at day 10 of diestrus, which corresponds to the implantation period, and the adhesion of *E. coli* to

endometrium was inhibited by the addition of D-Man at day 10 of diestrus (Ishiguro et al., 2007.

These facts indicate that specific sugar expressions on the mucosa would alter in the short-term which allows some bacteria to bind selectively. All things considered, it is suggested that sugar expressions on the mucosal surface would change not only site-specifically but also time-dependently, and these differences in sugar expressions indicate the possibility of the alteration of the settled bacterial species on the vaginal mucosa in pregnancy. Furthermore it is also speculated that the alteration of sugar expressions on the mucosa may allow pathogens to infect into the mucosa during limited periods in the genital tract.

Table 3. Lectins and sugar specificities

Lectin	Abbreviation	Sugar specificity
GalNAc		
Bandeiraea simplicifolia-I	BSL-I	α-GalNAc, α-D-Gal
Dolichos biflorus	DBA	α-D-GalNAc
Glycine maxi	SBA	α-D-GalNAc-3Gal
Sophora japonica	SJA	β-D-GalNAc
Vicia villosa agglutinin	VVA	D-GalNAc-3GalNAc
Gal		
Arachis hypogaea	PNA	β-D-Gal(1–3)D-GalNAc
Artocarpus integrifolia	JCA	β-D-galactopyranoside
Erythrina cristagalli	ECL	β-D-Gal(1–4)D-GlcNAc
Ricinus communis	RCA_{120}	β-D-Gal-4GlcNAc, D-Gal, D-GalNAc
GlcNAc		
Bandeiraea simplicifolia-II	BSL-II	D-GlcNAc
Datura stramonium	DSL	$(GlcNAc)_2$
Lycopersicon esculentum	LEL	$(GlcNAc)_{2-4}$
Solanum tuberosum	STL	$(GlcNAc)_{2-4}$
Succinylated WGA	s-WGA	$(GlcNAc)_n$
Triticum vulgaris	WGA	$(GlcNAc)_n$, NeuNAc
Glc/Man		
Canavalia ensiformis	Con A	α-D-Man, α-D-Glc
Lens culinaris	LCA	α-D-Man
Pisum sativum	PSA	α-D-Man
Fuc		
Ulex europaeus-I	UEA-I	α-L-Fuc-α-D-Gal-β(1–4)GlcNAc
OS		
Phaseolus vulgalis-erythroaggulutinin	PHA-E	bisected, triantennary N-glycans
Phaseolus vulgalis-leucoagglutinin	PHA-L	bisected, triantennary N-glycans

Fuc: fucose; Gal: galactose; GalNAc: N-acetylgalactosamine; Glc: glucose; GlcNAc: N-acetylglucosamine; Man: mannose; NeuNAc: N-acetylneuraminic acid; OS: oligosaccharide.

Table 4. Specific sugar expressions on the mouse vaginal mucosa

Lectin	D		P2		P7		P10		P13		P18	
	V	CC	V	CC	V	CC	V	CC	V	CC	V	CC
BSL-I	-	-	-	-	-	-	-	-	-	-	-	-
DBA	-	-	-	-	-	-	-	-	-	-	-	-
SBA	-	-	-	-	-	-	-	-	-	-	-	-
SJA	-	-	-	-	-	-	-	-	-	-	-	-
VVA	-	-	-	-	-	-	-	-	-	-	-	-
PNA	-	-	+	+	-	-	-	-	-	-	-	-
JCA	+	+	+	+	+	+	+	+	+	+	+	+
ECL	-	-	-	-	-	-	-	-	-	-	-	-
RCA_{120}	-	-	+	+	+	+	+	-	+	-	+	+
BSL-II	-	-	-	-	-	-	-	-	-	-	-	-
DSL	-	-	+	+	+	+	-	-	-	-	-	-
LEL	-	-	++	++	++	++	+	-	+	-	+	-
STL	-	-	+	+	+	+	-	-	-	-	-	-
s-WGA	-	-	-	-	-	-	-	-	-	-	-	-
WGA	++	++	+	+	++	++	+	+	+	+	+	+
Con A	+	+	-	-	+	+	+	+	+	+	+	+
LCA	-	-	-	-	-	-	-	-	-	-	-	-
PSA	-	-	-	-	-	-	-	-	-	-	-	-
UEA-I	-	-	-	-	-	-	-	-	-	-	-	-
PHA-E	+	+	-	-	+	+	+	+	+	+	+	+
PHA-L	-	-	-	-	-	-	-	-	-	-	-	-

D: diestrus; P: pregnant day; CC: vaginal portion of the cervical canal; V: vagina. ++: strong staining; +: moderate staining; -: negative staining.

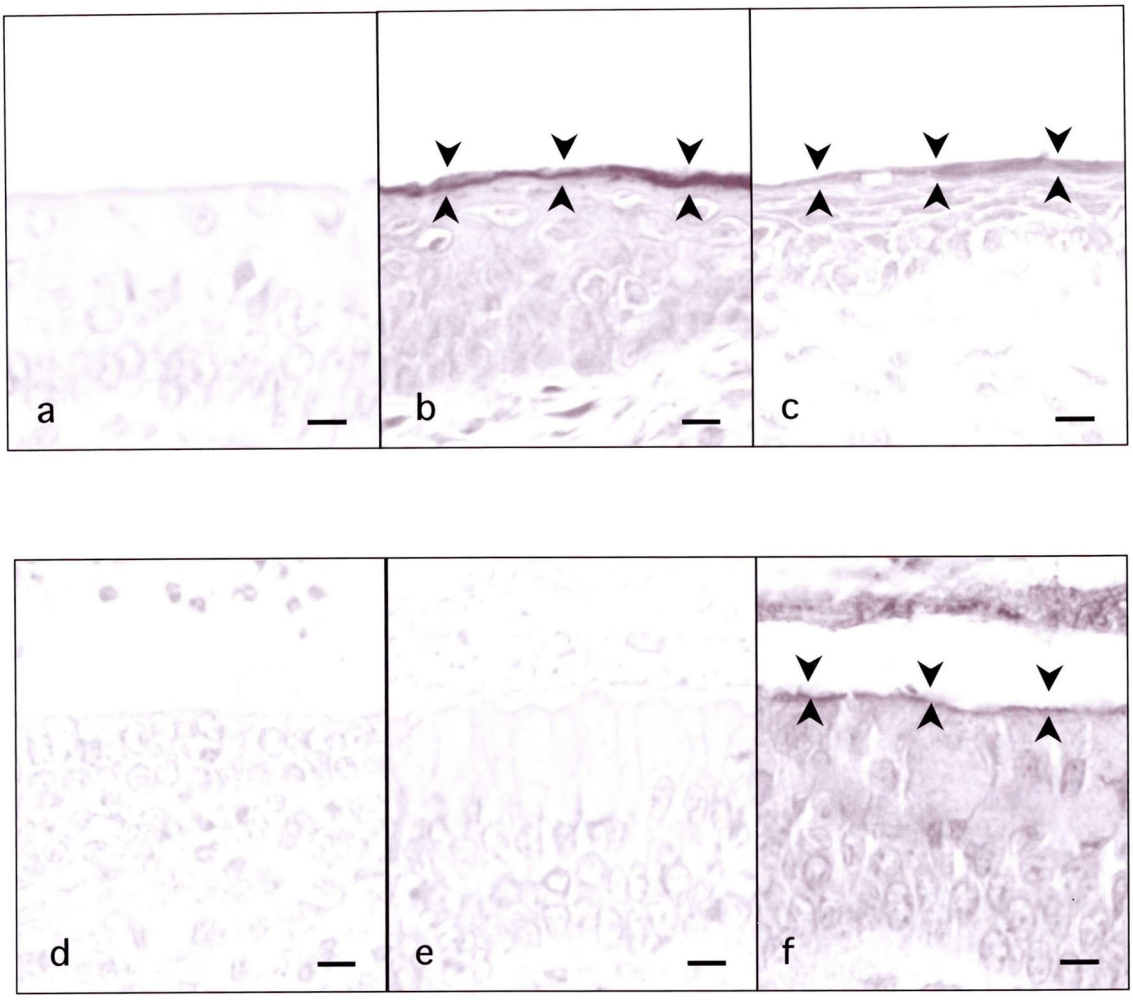

Fig. 3. Lectin staining patterns of RCA_{120} on the epithelium in the vaginal portion of the cervical canal. Apical surfaces of the epithelium are positive (arrowheads) for RCA_{120} on pregnant day (P) 2 (b), P7 (c) and P18 (f), but negative on diestrus (a), P10 (d) and P13 (e). Bar=10 μm.

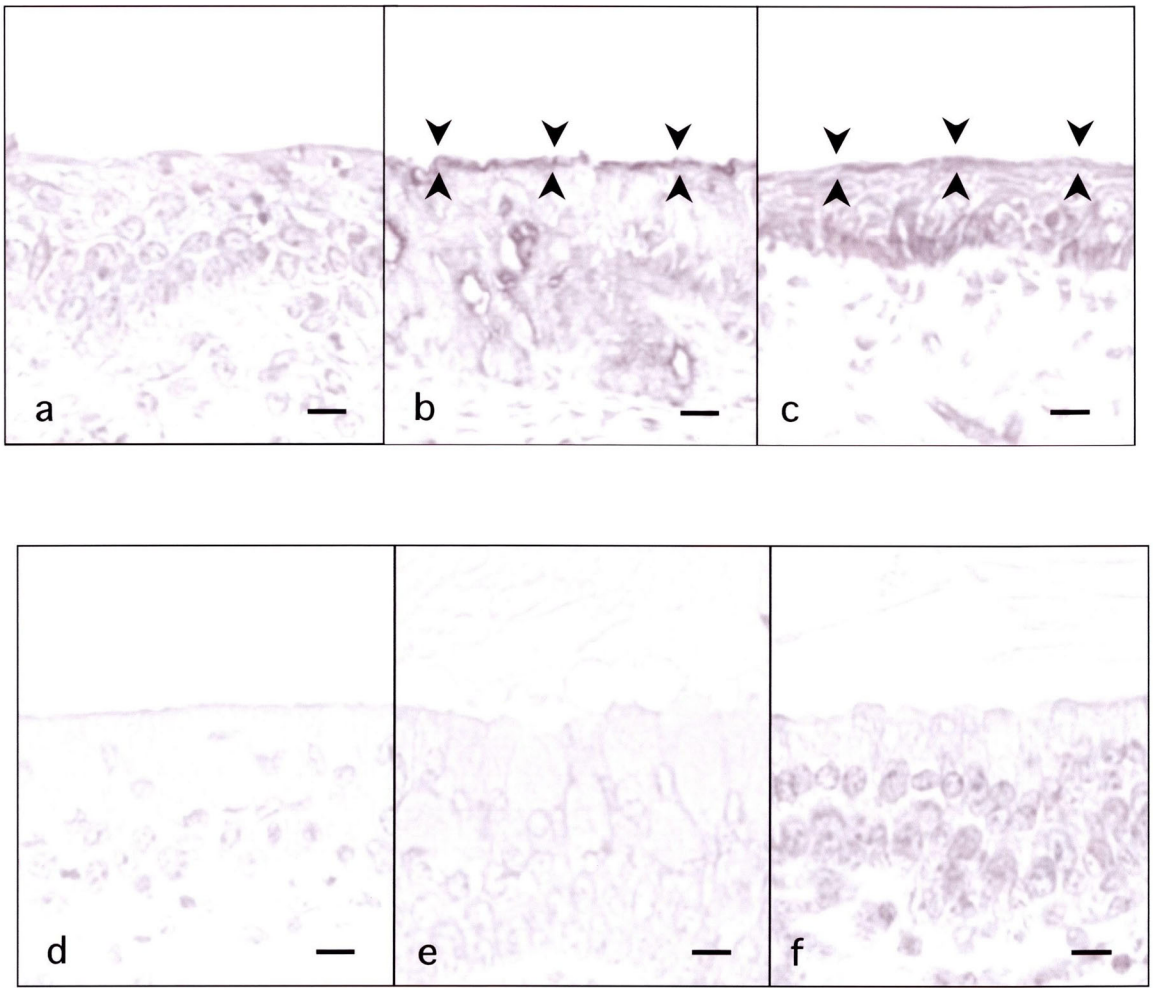

Fig. 4. Lectin staining patterns of STL on the epithelium in the vagina. Apical surfaces of the epithelium are positive (arrowheads) for STL on pregnant day (P) 2 (b) and P7 (c), but negative on diestrus (a), P10 (d), P13 (e) and P18 (f). Bar=10 μm.

IV. Chapter 3

Sugar Expression in the Mucosae of the Canine Uterus and Vagina during the Estrous Cycle and with Pyometra

Abstract

The pathogenesis of canine pyometra is still unclear, but bacterial infection of the endometrium, mediated by bacterial lectins, is suspected to induce pyometra. The aim of this study was to investigate sugar expression in the mucosae of the uterus and vagina of healthy dogs with normal estrous cycles and in dogs with pyometra, using a panel of lectins to investigate the pathogenesis of pyometra.

In pyometra dogs, the uterine and vaginal mucosae were positive for lectins that selectively bind to glucose or mannose, especially during days 7-10 and 30-40 of diestrus. These results suggest that temporal changes in sugar expression in the uterus and vagina present an opportunity for pathogens to infect the endometrium causing pyometra.

Keywords: Bitch; Lectins; Pyometra; Sugars; Uterus

Introduction

Pyometra occurs as a result of bacterial infection of the endometrium, but its cause is not fully understood (De Bosschere et al., 2001). Microbial

adhesion to the endometrial surface is mediated by bacterial lectins, which selectively combine with specific sugars on the superficial endometrium (Guarner and Malagelada, 2003). While sugar expression in the genital tract underlies the pathogenesis of pyometra, little is known about sugar expression in the canine endometrial and vaginal mucosae (Leitner et al., 2003).

The aim of this study was to compare the expression of sugars on the superficial epithelium of the uterus and vagina of healthy dogs and those with pyometra, using lectins which selectively bind to specific sugars (Gabius, 2001).

Materials and Methods

Samples were collected from nulliparous bitches of various breeds that were presented to veterinary clinics for routine ovariohysterectomy or to be treated for pyometra. Thirty-four tissue specimens (bitch age range 6 months-11 years; median: 1 year) were categorized as follows: immature (6 months-1 year); estrus (6 months-1 year); day 7-10 of diestrus (D7/10 - 6-11 months);

day 30-40 of diestrus (D30/40 - 9 months-1 year); young anestrus (1-2 years); aged anestrus (5-11 years), and pyometra (7-11 years). The stage of the estrous cycle was determined based on a history provided by the owner, serum progesterone concentrations and histological characteristics of the tissue specimens.

Specimens of uterus (from the cervix to the uterine ostium of the tube) and vagina (the vaginal portion of the cervical canal) were immersed in 10% formalin for at least 24 h. They were then embedded in paraffin and 4 μ m sections were pre-treated with serum-free protein block (Dako) for 1 h at room temperature. Sections were then incubated with 21 biotinylated lectins at a 1:5000 dilution (Vector Laboratories) for 18 h at 4 °C followed by peroxidase-labelled streptavidin (Nichirei) for 30 min at room temperature. Finally, sections were reacted with 3, 3-diaminobenzidine (Dako) and counterstained with hematoxylin. The lectin specificity was confirmed by pre-treatment with lectin solutions containing appropriate sugars and by incubation with the streptavidin solution or phosphate buffer. Differences of sugar expression

between categories of tissue specimens were evaluated by the Kruskal Wallis test (α = 0.05), using the Ekuseru-Toukei 2008 (Social Survey Research Information Co). The statistical analyses for the uterus and vagina and different sugar groups were performed separately.

Results and Discussion

Lectins can be classified into six groups on the basis of their sugar-binding properties, namely, N-acetylgalactosamine (GalNAc), galactose (Gal), N-acetylglucosamine (GlcNAc), glucose or mannose (Glc/Man), fucose (Fuc) and oligosaccharide (OS; Table 5). The sugar expressions in the specimens of uterus and vagina are presented in Table 6. In the GalNAc group, BSL-I and SBA were generally detected in the uterus throughout the estrous cycle, but SBA was not found in uterine specimens with pyometra. DBA and VVA were detected during D7/10 or D30/40, but specimens were negative during other phases of the estrous cycle. DBA was detected in vaginal specimens during young anestrus, aged anestrus and pyometra. In the Gal group, all lectins were

detected weakly or moderately in all uterine specimens. The same results were obtained in vaginal specimens without PNA. In the GlcNAc group, all uterine specimens were positive for most lectins, but BSL-II and s-WGA were not detected during pyometra. Vaginal specimens were positive for most lectins during pyometra without BSL-II and DSL.

In the Glc/Man group, ConA, LCA and PSA was distributed differently amongst different categories of uterine specimens ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively) and a stronger positive ConA expression was observed during D7/10 and pyometra (Fig. 5). In the vaginal specimens, ConA, LCA and PSA were also distributed differently amongst categories ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively), but stronger expressions were observed during D30/40 (Fig. 6). In the Fuc group, UEA-I was observed weakly or moderately in all categories of uterine specimens. The vaginal specimens were negative for UEA-I during estrus and D7/10. In the OS group, PHA-E was positive during D7/10 and pyometra, but PHA-L was negative during pyometra in the uterus.

In the vagina, PHA-E was positive during D7/10 and pyometra, and PHA-L was negative during both phases.

In the present study, lectins which selectively bind to glucose or mannose were detected in both the vaginal and uterine mucosa during D7/10, D30/40 and pyometra. These findings confirm those of Ishiguro et al. (2007), who demonstrated that the *E. coli* load in the endometrium increased at D10 and that bacterial adhesion to the endometrium was inhibited by the addition of D-mannose. Sugars in the genital mucosae play important roles not only for embryo recognition in pregnancy, but also in infection as a binding target for pathogens (Ohlsen et al., 2009). It has also been hypothesized that canine pyometra might occur as a result of an interaction between bacteria and the endometrium. The findings of the current study provide a possible mechanism of disease, which is similar to and further supported by work from Kida et al. (2006), who demonstrated that pyometra was observed particularly during the first half of diestrus, while bacteria were mainly detected in the uterus during proestrus and estrus. These reports suggest that the endometrium does not

constantly express specific sugars during the estrous cycle, thereby allowing bacteria to bind selectively.

Taken together, the current study and referenced work suggest that changes in sugar expression during the estrous cycle may affect bacterial colonization of the uterus and vagina, making the genital tract susceptible to infection during specific periods of the cycle. Since the potentially large number of pairwise comparisons prevented us from performing formal statistical tests between estrous cycle phases, only global tests were performed (i.e. Kruskal-Wallis tests). It has also been reported that the prevalence of pyometra differs with breed, age and geographic location (Egenvall et al., 2001), so the results reported here are not necessarily applicable across all populations of dogs. Further studies which take into account this variability are needed to elucidate the association between mucosal sugars and bacterial adherence.

Table 5. Lectins and sugar specificities

Lectin	Symbol	Sugar specificity
N-acetylgalactosamine group		
Bandeiraea simplicifolia-I	BSL-I	α-GalNAc, α-D-Gal
Dolichos biflorus	DBA	α-D-GalNAc
Glycine maxi	SBA	α-D-GalNAc-3Gal
Sophora japonica	SJA	β-D-GalNAc
Vicia villosa agglutinin	VVA	D-GalNAc-3GalNAc
Galactose group		
Arachis hypogaea	PNA	β-D-Gal(1–3)D-GalNAc
Artocarpus integrifolia	JCA	β-D-galactopyranoside
Erythrina cristagalli	ECL	β-D-Gal(1–4)D-GlcNAc
Ricinus communis	RCA_{120}	β-D-Gal-4GlcNAc, D-Gal, D-GalNAc
N-acetylglucosamine group		
Bandeiraea simplicifolia-II	BSL-II	D-GlcNAc
Datura stramonium	DSL	$(GlcNAc)_2$
Lycopersicon esculentum	LEL	$(GlcNAc)_{2-4}$
Solanum tuberosum	STL	$(GlcNAc)_{2-4}$
Succinylated WGA	s-WGA	$(GlcNAc)_n$
Triticum vulgaris	WGA	$(GlcNAc)_n$, NeuNAc
Glucose/mannose group		
Canavalia ensiformis	ConA	α-D-Man, α-D-Glc
Lens culinaris	LCA	α-D-Man
Pisum sativum	PSA	α-D-Man
Fucose group		
Ulex europaeus-I	UEA-I	α-L-Fuc-α-D-Gal-β(1–4)GlcNAc
Oligosaccharide group		
Phaseolus vulgalis-erythroaggulutinin	PHA-E	Bisected, triantennary N-glycans
Phaseolus vulgalis-leucoagglutinin	PHA-L	Bisected, triantennary N-glycans

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNac, N-acetylneuraminic acid.

Table 6. Sugar expressions on the mucosal surface of the uterus and vagina

Lectin	Immature		Estrus		D7/10		D30/40		Young anestrus		Aged anestrus		Pyometra	
	U	V	U	V	U	V	U	V	U	V	U	V	U	V
GalNAc														
BSL-I	3	2	3	3	3	1	3	3	3	3	3	3	2	1
DBA	2	1	1	1	3	1	1	1	1	3	1	3	1	2
SBA	3	1	3	3	3	1	3	2	3	2	3	2	1	2
SJA	3	1	1	1	2	1	1	1	3	2	3	2	1	1
VVA	2	1	1	1	3	1	3	1	1	1	1	1	1	1
Gal														
PNA	3	1	2	3	3	1	3	2	3	2	3	2	2	1
JCA	3	1	3	2	3	3	3	3	3	3	3	3	3	3
ECL	3	3	2	2	2	1	3	2	3	3	3	3	2	2
RCA_{120}	4	3	3	3	4	3	4	4	4	4	4	4	4	3
GlcNAc														
BSL-II	1	1	1	1	2	1	1	1	1	1	1	1	1	1
DSL	3	1	3	2	3	2	3	3	3	3	3	2	2	1
LEL	3	3	3	3	3	3	3	3	3	3	3	3	3	2
STL	3	3	3	2	3	2	3	3	3	3	3	3	3	3
s-WGA	3	3	1	1	3	1	3	3	3	3	3	3	1	3
WGA	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Glc/Man														
ConA	1	1	1	1	3	2	2	3	2	2	2	2	3	2
LCA	2	2	2	2	3	2	3	3	2	2	2	2	2	2
PSA	1	1	1	1	3	2	2	3	2	1	2	1	2	2
Fuc														
UEA-I	3	3	2	1	2	1	2	2	3	3	3	3	3	3
OS														
PHA-E	1	1	1	1	3	3	2	4	3	3	3	2	3	3
PHA-L	2	2	1	1	3	1	1	1	1	1	1	1	1	1

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; OS, oligosaccharide; U, uterus; V, vagina; D7/10, days 7-10 of diestrus; D30/40, days 30-40 of diestrus; 4, strong reaction; 3, moderate reaction; 2, weak reaction; 1, negative reaction. Values are presented as the medians of samples (n = 5, immature; estrus, D30/40; young anestrus, aged anestrus and pyometra; n = 4, D7/10).

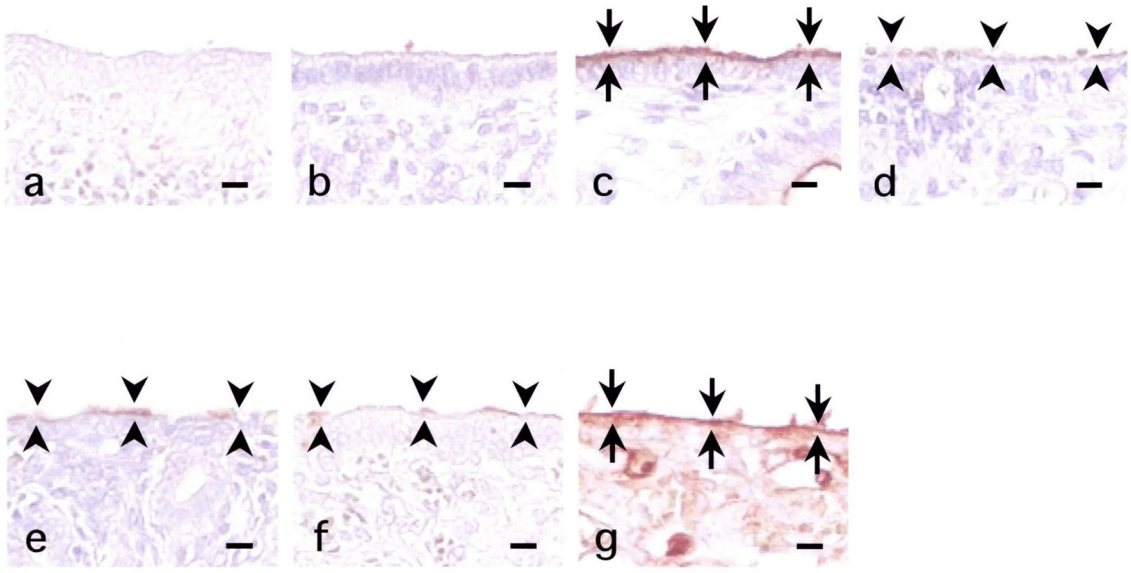

Fig. 5. Lectin-staining patterns for ConA in the uterine mucosa. A stronger positive expression (arrow) was observed during days 7-10 of diestrus (c) and pyometra (g) in apical surfaces of epithelial cells, and a weak expression (arrowhead) was seen during days 30-40 of diestrus (d), young anestrus (e) and aged anestrus (f). The apical surfaces are negative in the immature (a) and estrus b) specimens. Bar represents 10 μm.

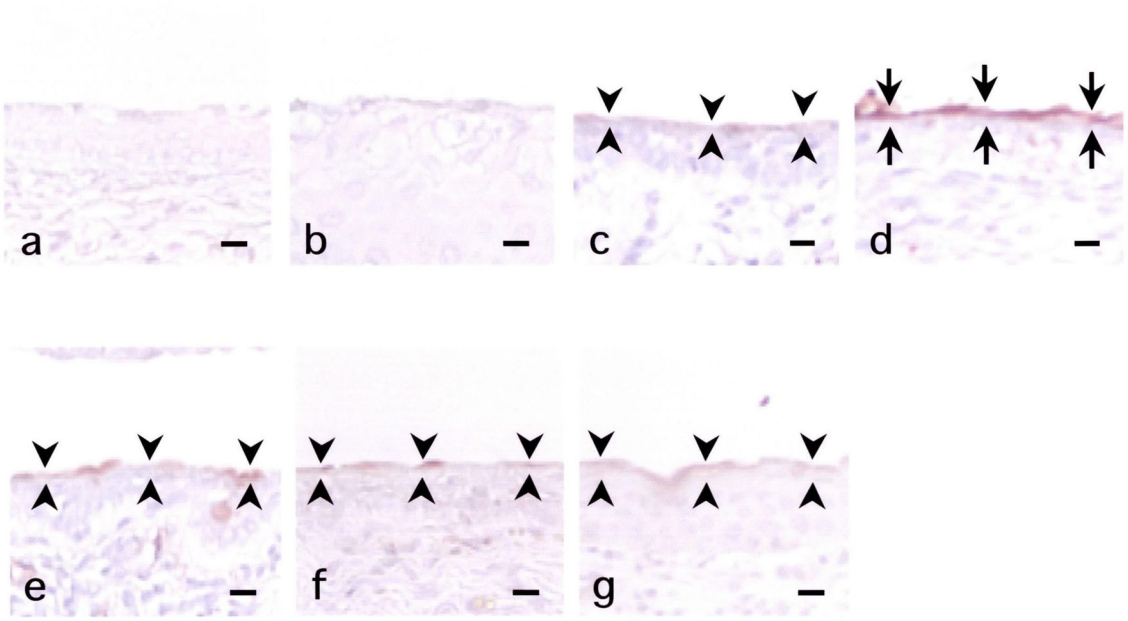

Fig. 6. Lectin-staining patterns for ConA in the vaginal mucosa. A stronger positive expression (arrow) was observed during days 30-40 of diestrus (d) in apical surfaces of epithelial cells, and a weak expression (arrowhead) was seen during days 7-10 of diestrus (c), young anestrus (e), aged anestrus (f) and pyometra (g). The apical surfaces are negative in the immature and estrus (a and b) specimens. Bar represents10 μ m.

V. Summary and Conclusion

Microorganisms, such as the bacteria, which reside on the host body as the flora, are known to be beneficial for host fitness. However, under certain conditions some of them acquire virulence-associated genes, become pathogenic and cause disease. In order to cause disease, bacteria must access to the host body to colonize the appropriate niche. To colonize host mucosal surfaces, microorganisms have developed the capacity to produce surface molecules which enable them to adhere to the host cells and tissues. Adhesion protects bacteria against natural cleaning mechanisms of the host, such as peristalsis of the intestine, coughing, airflow in the respiratory tract or the flow of urine through the urinary tract, and provides better access to the sources of nutrition. It also facilitates the delivery of toxic agents and invasion of the bacteria into host tissues and cells.

Many of the adhesive surface molecules expressed by bacteria are carbohydrate-binding proteins (lectins). Lectins can bind to the complementary carbohydrate receptors of the host cell membrane glycoproteins or glycolipids

and determine the species specificity of pathogens, and their preference for certain host cell or tissue types. For example, some kinds of fimbriated strains of enterotoxigenic *E. coli* can cause diarrhea in piglets, but not in adult pigs or humans. Alternatively, host cells also express various cell surface molecules that bind carbohydrates present on the surface of other cells or bacteria. The specific interactions between lectins and glycoproteins are also crucial in cell to cell or cell to matrix interactions, signaling, differentiation and development. Several other types of infectious agents such as viruses, fungi (e.g. *Candida albicans*) and amoebal parasite (e.g. *Acanthamoeba*) also require carbohydrate-mediated adherence for infection.

On the other hand, it has been reported that bacteria activation induces the pregnancy failure, such as fetal resorption, abortion and preterm birth in the genital mucosa of mammals, and it has been also reported that bacterial infection of the endometrium would induce pyometra in bitches. Moreover, recently it has been speculated that the adhesion of microbes to mucosae is mediated by bacterial lectins which selectively combine with specific

glycoconjugates on the cell surface. Actually, mouse models of intrauterine inflammation demonstrated that administration of LPS or Gram-negative bacteria induced preterm birth, and the administration of CpG DNA induced inflammatory response in the genital mucosa, resulting in fetal resorption, abortion and preterm birth. In addition to this, the glycocaryx of the epithelial cells has been known to be a binding site for microorganisms in the mucosa of the mammalian intestine and trachea. From these findings, I planned to investigate the sugar expressions on mucosae of the uterus and vagina of the pregnant mice and bitches with pyometra in the present thesis, by using a panel of lectins, in order to clarify the pathogenesis of the canine pyometra, and consequently elucidate the mechanism of pregnancy failure.

Firstly, expression patterns of sugars on the mucosal surface of the uterus in pregnant mice were investigated. In the uterine mucosa, the GlcNAc group tended to express a positive reaction before pregnant day 10, but the glucose/mannose group generally expressed a positive reaction after pregnant day 10. On the other hand, the fucose group expressed a negative reaction

during all periods in pregnancy. These findings were approximately the same on both the mesometrial and anti-mesometrial side of the uterus. These differences of sugar expression reflect the functional change of the mucosa during pregnancy, and the alteration of sugar expression may give a chance for pathogens to infect in the uterus with limited periods.

Secondly, sugar expressions were examined on the epithelium of both the middle portion of the vagina and the vaginal portion of the cervical canal (CC) in pregnant mice. N-acetylglucosamine was positive before pregnant day 7, but negative on both the vagina and the CC after pregnant day10 and at diestrus. In addition, some differences in sugar expressions were seen between them. These results suggest that sugar expressions on the mucosal surface would change not only site-specifically but also time-dependently, and these sugar differences indicate the possibility of the alteration of the settled bacterial species on the vaginal mucosa in pregnancy.

Finally, I investigated sugar expressions on mucosae of the uterus and vagina with the normal estrous cycle and pyometra in bitches. Lectins which

selectively bind to glucose or mannose were positive on uterine and vaginal mucosae especially at days 7-10 and 30-40 of the diestrous stage and the pyometra. These findings confirm the findings that the *E. coli* load in the endometrium is increased at D10 and that bacterial adhesion to the endometrium is inhibited by the addition of D-mannose. It has been hypothesized that canine pyometra might occur as a result of an interaction between bacteria and the endometrium, and changes of sugar expressions would consequently make it possible for pathogens to infect the endometrium period-dependently in the pyometra.

In conclusion, the present study indicates that sugar expressions on the mucosal surface change time-dependently, which makes it possible for pathogens to infect the mucosa of the vagina and endometrium and consequently induces various failure of pregnancy.

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