Notes

Presence of surfactant proteins in the uteri and placentae of pregnant mares

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Running head: SURFACTANT PROTEINS IN MARE UTERI, PLACENTAE
ABSTRACT

Immunohistochemical investigations of the expression of surfactant protein A (SP-A) and surfactant protein D (SP-D) in the uterine and placental tissues of 13 pregnant mares were performed using anti-horse monoclonal primary antibodies. Strong positive reactions for both SP-A and SP-D were observed in the trophoblasts in the microcotyledons of the placentae at 182 to 314 days of gestation; in uterine glandular epithelial cells, faint-to-weak reactions were observed during gestation. This study describes, for the first time, the changes in the SP-A and SP-D expression levels in the endometrium of mares during gestation; the SP-A and SP-D expression levels increased after the second trimester of gestation.

KEY WORDS: mare, placentae, surfactant protein, uteri
Currently, four surfactant proteins have been identified in the lungs [6], namely, the hydrophilic surfactant proteins A and D (SP-A and SP-D) and the hydrophobic surfactant proteins B and C (SP-B and SP-C). These proteins play an important role in the pulmonary surfactant function. In particular, SP-B and SP-C are able to fuse with phospholipid membranes, which are needed for pulmonary surfactants to express their surfactant activity [40]. In contrast, SP-A and SP-D belong to the C-type lectin (collectin) family, which binds with carbohydrates in the presence of calcium ions. These proteins have host defense actions [14], bind with viruses and bacteria to cause aggregation [8, 9, 38, 39, 42], promote the binding activity of macrophages, and promote phagocytosis by acting as opsonins [5, 20, 28]. Moreover, they exert a direct antimicrobial effect by binding with bacteria and changing the permeability of their cell membranes [41]. SP-A- and SP-D-knockout mice have been shown to be susceptible to infection [18, 19]. SP-A and SP-D have also been shown to be involved in viral neutralization, clearance of bacteria, fungi, and apoptotic and necrotic cells, downregulation of allergic reactions, and resolution of inflammation [15]. Thus, SP-A and SP-D can be considered factors that play important roles in host defense in the bronchoalveolar system. Moreover, they have been found in the amniotic fluid, amnion, and trophoblasts. Findings such as the increase in the levels of these surfactant proteins in the amniotic fluid as delivery approaches [23] have garnered attention from a reproductive standpoint. SP-A is an important protein in the fetal lungs and amniotic fluid, where it can be detected at 20 weeks of gestation [3, 29]. Fetal SP-A has been suggested to serve as the trigger for delivery in mice [4]. Furthermore, SP-D expression has been observed in the ovaries and uteri of humans and mice [1, 17, 21, 26, 27], and it has been shown to intensify during the luteal phase in the endometrium in non-pregnant humans [17]. SP-D has also been shown to prevent Chlamydia trachomatis infection, a common reproductive organ infection in humans [26]. The above findings suggest that these proteins play a role in preventing reproductive organ infections.
during implantation and pregnancy. However, while its expression has been reported in non-pregnant horse uteri [12], there have been no studies of its presence in the placenta; thus, their role in maintaining pregnancy is unclear.

Therefore, considering that SP-A and SP-D may be necessary to prevent reproductive organ infections and maintain pregnancy, we conducted an immunohistochemical study to examine the expression of these proteins in the uteri and placentae of horses.

Tissue samples

Uteri and placenta tissue samples from pregnant mares were obtained from euthanized mares used in other studies [35-37]. The uteri and placentae tissues were obtained from 13 clinically normal pregnant Thoroughbred and Anglo-Arabian mares at 57 to 314 days of gestation (day 0 being the day of last mating). The mares were euthanized using an overdose of thiopental sodium (Ravonal; Tanabe Pharmaceutical Co. Ltd., Osaka, Japan) and suxamethonium chloride (Succine; Yamanouchi Pharmaceutical Co. Ltd., Osaka, Japan) after the intravenous administration of medetomidine (Domitor; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) prior to tissue recovery. After fixing with 4% paraformaldehyde in 0.01 M phosphate buffered saline, the samples were embedded in paraffin. All procedures were performed in accordance with the guidelines established by the Rakuno Gakuen University for the use of laboratory animals.

Antibodies

The primary antibodies against SP-A (SP-A 08 IgM 1 mg/ml) and SP-D (αD13 IgG 500 μg/ml) were mouse monoclonal antibodies for horse SP-A and mouse monoclonal antibodies for horse SP-D. As previously reported, these antibodies specifically recognize SP-A and SP-D, respectively [10], and have also been used for performing immunohistochemical analyses of the genital tract of mares [12].
**Immunohistochemistry**

The formalin-fixed uteri were sliced to yield 5-mm-thick sections and washed with running water for 10 min. Further, after dehydration using an alcohol series (70%, 80%, 90%, 95%, and 100%) and dewaxing using xylene, the samples were embedded in paraffin. The paraffin-embedded tissue samples were sectioned at a thickness of 6 µm using a microtome, placed on silane-coated slides, stretched, and dried for 1 h at 45°C.

Immunostaining of SP-A and SP-D was performed using an enzyme antibody technique with streptavidin and biotin binding (LSAB). A Histofine SAB-PO(M) kit (code: 424021, Nichirei Biosciences Inc., Tokyo, Japan) and DAB matrix kit (code: 425011, Nichirei Biosciences Inc.) were used for immunostaining. In addition, phosphate buffered saline (PBS) (-) powder diluted with ultrapure water was used as the phosphate buffer during staining. After the thorough deparaffinization of the paraffin slices using xylene (5 min, 2 times), the samples were immersed in a graded series of alcohol solutions (100%, 90%, 80%, and 70%; 5 min each), washed with tap water, and then washed with ultrapure water. After washing, the tissue slices were treated with 3% hydrogen peroxide solution for 5 min at room temperature (20°C) for the inactivation of endogenous peroxidases and washed thoroughly with PBS. Fresh rabbit serum (10%) was added to the tissue slices, and the reaction was allowed to occur for 15 min at room temperature. After the reaction, the primary antibodies for each antigen were diluted by 250-fold in PBS; the tissue slices were treated with these primary antibody solutions overnight at 4°C. Post incubation, the tissue slices were washed with PBS, and then treated with the secondary antibodies (biotin-labeled anti-mouse IgG, IgM, and IgA antibodies (10 µg/ml): Histofine SAB-PO(M) kit) for 30 min at room temperature. Next, the tissue slices were washed with PBS and treated with an enzyme-linked reagent (peroxidase-labeled streptavidin) for 5 min at room temperature. Next, the tissue slices were washed with PBS and treated with the DAB coloring solution for 7 min for the visualization of the various
areas where the tissue–antibody immunoreactions had occurred. After the coloring reaction, the tissue slices were washed with tap water and ultrapure water and nuclear staining was performed with hematoxylin (3 min); the tissues were then washed again with ultrapure water. Next, the tissue slices were dehydrated using a graded series of alcohol solutions (70%; 3 seconds; 80%, 90%, 100%, 100%, and 100% for 2 min each) and dewaxed using xylene (twice; 5 min each), after which they were mounted using a xylene mounting medium and observed under a light microscope. The negative control was established by replacing the primary antibody with mouse IgM (SC-3881, Santa Cruz Biotechnology, Dallas, TX, USA) for SP-A and mouse IgG (SC-2025, Santa Cruz Biotechnology) for SP-D. Immunostaining was performed using horse lung samples as the positive and negative controls.

The results of the presence of SP-A and SP-D in the uteri and placentae of pregnant mares (13 cases) are summarized in Table 1. Faint (Fig. 1a–d) to weakly positive (Fig. 1e–h) reactions were observed in the uterine gland epithelial cells. Negative (Fig. 1a–d) to weakly positive (Fig. 1e–h) reactions were observed in the loose connective tissue cells around the uterine glands. The positive reactions in the trophoblasts intensified as pregnancy progressed (Fig. 1). While the positive reactions were faint in the first trimester, strong positive reactions were observed as the microcotyledons started to form in the second trimester (Table 1). Differences in the degree of staining in the uteri and placentae of pregnant mares were not related to the sex of the fetus.

The present study demonstrated the presence of SP-A and SP-D in pregnant mare uteri and placentae. Kankavi et al. [12] studied 5 non-pregnant mares and reported the expression of SP-A and SP-D in the endometrium and epithelium. SP-A and SP-D expression in human endometrial tissues was reported to be stronger in the luteal phase
than in the follicular phase during the menstrual cycle and was present in placental
trophoblasts throughout pregnancy [17, 23]. In the present study, SP-A and SP-D were
observed in the trophoblasts in the microcotyledon during pregnancy, which is consistent
with the above findings in humans [17, 23].

Previous studies on humans and mice have shown that SP-A is produced in many
tissues of the reproductive canal, including the embryonic membrane [7, 16, 23, 34], villi,
and placental trophoblasts [31, 34]. Condon et al. [4] found that the SP-A levels in the
amniotic fluid increased with the increase in the duration of pregnancy. They also found
that administering SP-A within the mouse fetal amnion on day 15 of pregnancy caused
premature birth or parturition and that the administration an SP-A-neutralizing antibodies
in the amnion extended the pregnancy. Therefore, surfactant proteins, especially SP-A,
have been shown to be directly associated with labor induction in mice [4]. Snegovskikh
et al. [32] found that SP-A produced by the endometrium/deciduous membrane in humans
hindered prostaglandin F$_{2\alpha}$ production without affecting the production of other
inflammatory mediators or angiogenesis factors. Furthermore, these authors suggested
that SP-A in the deciduous membrane plays an important role in regulating prostaglandin
production in the uterus. In contrast, SP-D expression has been observed in human and
mouse female reproductive organs, placenta, amniotic fluid, and ovaries [1, 17, 26, 27];
moreover, SP-D in the endometrium has been suggested to prevent intrauterine infections
during implantation and pregnancy [17, 26, 27]. Furthermore, estrogen positively
regulates SP-D expression in the mouse uterus [13] and may regulate SP-D gene
expression [27]. Progesterone, along with estrogen, synergizes SP-D expression, but
when administered alone results in the negative regulation of SP-D expression [13].

Blood progesterone levels in pregnant horses are further increased by the accessory
corpus luteum from around 35 days of gestation, but a sharp increase in the estrogen levels
begins at around 90 days of gestation. Therefore, the placenta on the 57th day of
pregnancy was in a progesterone-significant state, and the expression of SP-D may have been weak.

SP-D mRNA levels in the mouse uterus have been reported to be hormonally regulated, with the highest levels being present at the estrus phase, and the lowest levels, at the diestrus phase [27]. Unlike SP-D, human SP-A is localized in both premenopausal and postmenopausal vaginal stratified squamous epithelium and vaginal lavage fluid, and SP-A immunoreactivity in the premenopausal vaginal epithelium is not cyclical [20]. This suggests that SP-A is constitutively expressed in the vaginal epithelium in humans and that its levels are not affected by ovarian hormones. Both SP-A and SP-D can be detected in the human amniotic fluid as early as 26 weeks of gestation; by 40 weeks of gestation, the SP-A levels rise dramatically, but the SP-D levels are not very noticeable [23]. Salminen et al. [30] reported that the SP-A protein expression was higher in the choriodecidua than in the amniotic membrane, and SP-D was preferentially expressed in the amnion. Thus, there are several species-specific differences in the expression of SP-A and SP-D in the female reproductive tract. In this study, the expression intensity of SP-D in the trophoblasts increased after 165 days of gestation. Since estrogen levels in pregnant mares generally increase between 5 to 10 months of gestation, estrogen may have led to the increase in SP-D expression. It has been reported that the human myometrium is a source of SP-A and SP-D, and triggers contractility of the uterus by inducing the expression of contraction-associated protein genes such as oxytocin receptors and augmenting the secretion of proinflammatory cytokines [33]. Since it is also involved in uterine contraction in this way, it is expected that the expression of SP-A and SP-D will be further enhanced immediately before foaling.

SP-A and SP-D may be involved in the recognition and clearance of pathogens from the fetal membrane and amniotic fluid via amniotic membrane cells or macrophages in the decidua [22]. Montalbano et al. [24] observed the reduced expression of
inflammation-related genes that delay delivery in SP-A- and SP-D-knockout mice and reported that this affected the time until delivery in their reproductive histories. The immunoregulatory functions of SP-A and SP-D have also been observed to regulate cytokine production within the uterine environment [25]. Inhibition of tumor necrosis factor - α production by SP-A in lung macrophages can hinder the premature activation of the prostaglandin cascade. Hence, when this inhibition of the expression of pro-inflammatory cytokines occurs pregnancy, it leads to the regulation of the onset of labor [2]. These findings indicate that the expression levels of SP-A and SP-D in the uterus during pregnancy play important roles in preventing intrauterine infections, as well as in maintaining pregnancy. Furthermore, both SP-A and SP-D in mare trophoblasts may play similar roles in uterine defense mechanisms and in maintaining pregnancy. The fetal placenta primarily consists of the chorion, and the trophoblasts are important sites on the fetal side for exchanging oxygen and nutrients received from the mother. Trophoblasts have also been reported to secrete substances essential for fetal survival [11]. Therefore, SP-A and SP-D in horse trophoblasts may be essential for fetal development.

Although this study was limited to the analysis of the localization of SP-A and SP-D in the uterus and placenta during pregnancy, additional studies are necessary to detect SP-A and SP-D in the amniotic fluid of pregnant mares and to measure changes in the blood levels of these proteins to further clarify their physiological roles in maintaining pregnancy.

In conclusion, to the best of our knowledge, the present study describes, for the first time, the changes in the endometrial SP-A and SP-D expression during pregnancy in mares and demonstrates the existence of these proteins in the placenta during pregnancy. In the placenta of mares during pregnancy, SP-A and SP-D, which play a role in the host defense against viruses and bacteria, may help maintain healthy pregnancies by preventing intrauterine infections, regulating the intrauterine environment, and playing
other important roles such as immunomodulation.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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Table 1. Staining intensity of immunostaining for SP-A and SP-D in pregnant mare uteri and placentae

Fig. 1. Immunohistochemical results of SP-A and SP-D in the uterus and placenta of pregnant mares. A positive control (posi. con.) and negative control (nega. con.) was set up using horse lung tissues; (a, b) Uterus and placenta on the 57th day of pregnancy: faint positive reactions of the uterine gland epithelial cells (arrows) and faint positive reactions of the trophoblast cells invading the endometrium (arrowheads); (c, d) Uterus and placenta on the 131st day of pregnancy, moderate (SP-A) and weak (SP-D) positive reactions (arrow) in trophoblast cells; (e, f) Uterus and placenta on the 182nd day of pregnancy, The strong positive reaction of trophoblast cells of the microcotyledon (arrow) and weak positive reaction of the uterine gland epithelial cells (arrowhead); (g, h) Uterus and placenta on the 314th day of pregnancy, The strong positive reaction of trophoblast cells of the microcotyledon (arrow) and weak positive reaction of lyophobic connective tissue around the uterine gland (arrowhead) (Eosin staining). The scale bar represents 50 µm.
Table 1. Staining intensity of immunostaining for SP-A and SP-D in pregnant mare uteri and placentas in the immunostaining analysis.

| Mare number | Days of gestation | Fetal sex | Uterine glandular epithelial cells | Connective tissue cells around the uterine glands | Trophoblasts |
|-------------|-------------------|-----------|-----------------------------------|-------------------------------------------------|--------------|
|             |                   |           | SP-A | SP-D | SP-A | SP-D | SP-A | SP-D | SP-A | SP-D |
| 1           | 57                | Female    | ±    | ±    | -    | -    | ±    | ±    |       |       |
| 2           | 110               | Female    | ±    | ±    | -    | -    | ++   | ++   |       |       |
| 3           | 131               | Male      | ±    | ±    | -    | -    | ++   | +    |       |       |
| 4           | 142               | Female    | +    | +    | +    | ±    | ++   | ++   |       |       |
| 5           | 144               | Male      | ±    | ±    | ±    | ±    | ++   | +    |       |       |
| 6           | 165               | Female    | ±    | +    | ±    | +    | ++   | +++  |       |       |
| 7           | 182               | Male      | +    | +    | +    | +    | +++  | +++  |       |       |
| 8           | 190               | Male      | +    | +    | +    | +    | +++  | +++  |       |       |
| 9           | 198               | Female    | +    | +    | -    | +    | +++  | +++  |       |       |
| 10          | 208               | Male      | +    | +    | +    | +    | +++  | +++  |       |       |
| 11          | 227               | Male      | +    | +    | +    | +    | +++  | +++  |       |       |
| 12          | 270               | Female    | +    | +    | +    | +    | +++  | +++  |       |       |
| 13          | 314               | Male      | +    | +    | +    | +    | +++  | +++  |       |       |

- : negative, ± : faint positive, + : weak positive, ++ : moderate positive, +++ : strong positive.
Fig. 1.

SP-A

SP-D