



Morphological Changes by Matrix Metalloproteinase Activation and Apoptosis in the Endometrium of Pregnant Water Deer and Spotted Deer

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ABSTRACT

Water deer and sika deer are animals that ovulate without clear signs of estrus, similar to mon-ovulatory species. They are maintained as 'silent ovulations' and have a high probability of causing fetal miscarriage during pregnancy. Therefore, this study analyzed the difference in endometrial remodeling between water deer and sika deer and used this as primary data for wild deer restoration. We analyzed the activity of pregnancy-associated plasma protein A (PAPP-A) and matrix metalloproteinases (MMPs) and the association between vascular endothelial growth factor (VEGF) and apoptosis in uterine tissues during pregnancy in sika deer near the Korean peninsula and in water deer in Korea. Our results showed that the development of the endometrial villus cytotrophoblast zone in sika deer was significantly higher than that in water deer. However, the Ca²⁺ deposition rate was higher in the villus cytotrophoblasts of water deer. In addition, the expression of insulin-like growth factor1 receptor (IGF1-r) and PAPP-A in sika deer was high in the major binucleate cells (BNCs) of villus cytotrophoblasts, and the activity of MMPs in utero was high in water deer. However, VEGF and apoptosis in BNCs, which mainly constitute the endometrium, were greatly increased in sika deer, suggesting that endometrial changes in sika deer are more dynamic than in water deer.

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Key words

Sika deer, Water deer, Apoptosis, Cell remodeling, Endometrium

INTRODUCTION

Changes in the endometrium are important for the development and growth of the fetus during pregnancy and the activation of nutrition and maternal transitional

immunity. In particular, Artiodactyla with caruncle placenta, undergo continuous changes in the uterus and achieve successful maturation of the endometrium for the maintenance of pregnancy. In Artiodactyla, three important types of trophoblast cells [uninucleate trophoblast cells (UTCs), binucleate trophoblast giant cells (TGCs), and hybrid fetomaternal syncytial plate cells (TNCs)] of the fetus, which are connected to the placenta and uterus, differentiate into trophoblast binucleate cells (BNCs) according to the gestation period and change the characteristics of the placenta (Wooding *et al.*, 1997). This trophoblastic differentiation phenomenon is characterized by the close cooperation of the endocrine hormones estrogen and progesterone, which affect the uterus and regulate pregnancy and delivery (Kim *et al.*, 2014; Ott,

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2019; Topaloğlu and Aydın Ketani, 2021).

Thus, the placenta exhibits functional differences according to its shape and histological structure. It is located in the BNCs fetal trophoctoderm, migrates through tight junctions, and trans-forms the uterine epithelium into the fetal syncytium (Wooding, 1984; Wooding *et al.*, 1993). In mammals, pregnancy-specific protein B is secreted from the placental lactogen, and BNCs in the fetal-maternal syncytium are mainly secreted near the maternal blood vessels (Wooding *et al.*, 1997; Wooding, 1984). Conversely, in cows and deer, trinucleate cells produced by BNCs are reabsorbed by the trophoctoderm after secretion (Wooding, 1984, 1992; Wooding *et al.*, 1993, 2018). The fusion of BNCs with the uterine epithelium is essential for facilitating the transfer of fetal hormones and proteins to the maternal circulation (Wooding, 1993; Wooding *et al.*, 2005). Thus, the uterus changes rapidly during pregnancy to form a normal placenta. However, it has been postulated that water deer and sika deer, which are different from other Artiodactyla species, may exhibit differences in placental formation and uterine shape (Sohn and Kimura, 2012).

Previous studies have shown water deer and sika deer to have similar breeding patterns, and estrus and gestation are very similar. These two species are known to undergo at least five to eight cycles of follicular development during estrus. Like monovulatory species, 'silent ovulations' may occur (Asher *et al.*, 1999; Asher, 2011). According to a study by Sohn *et al.* (2021) the placenta of the Korean water deer is classified as oligo-cotyledonary, similar to other Cervidae families with six to eight placentae (Hamilton *et al.*, 1960) with villi. It is well-developed, showing a distinct polycotyledonary in each placenta. The overall shape of the sika deer placenta is similar to that of water deer. However, the state of fetal villi branching in the convex placenta is different for each Cervidae family (Hamasaki *et al.*, 2001; Chen *et al.*, 2015; Kotlarczyk *et al.*, 2021). Although the morphological findings of the water deer and sika deer uterus and placenta are similar in shape, it can be confirmed that there is a difference in the shape of the uteroplacental junction that spreads from the uterus to the placenta.

In the past, sika deer were very common in Korea, but are now officially nationally extinct (Whitehead, 1993). In addition, the differences in tissue reorganization, which is very important for the morphological changes of the uterus and placenta necessary for the reproductive menstrual cycle and maintenance of pregnancy, between water deer and sika deer have not yet been identified. Therefore, this study aimed to determine whether there is a difference in tissue reconstruction in the uteri of water deer and sika deer during pregnancy and to analyze the

morphological differences accordingly to investigate the reproductive physiological characteristics of extinct Korean sika deer.

MATERIALS AND METHODS

Animals

The Korean water deer used in this experiment were captured between November and January in Ansansi, Gyeonggi-do, Korea, in accordance with the Wildlife Protection and Management Act (Article 19, Paragraph 1 of the Act), and the uterus of a water deer whose early embryo was confirmed was collected. In the case of sika deer, an individual believed to be a Korean sika deer inhabiting the Primorskaya State Agricultural Academy and the adjacent area of Primorskaya Oblast in North Korea was captured. The uterus was then harvested from the sika deer, in which an early fetus was identified (MOU with the Primorskaya State Agricultural Academy; date: 2014.11.25). The uteri were collected after selecting total eight animals from water deer (four animals) and sika deer (four animals), and the collected uteri were stored at -80°C until experimental analysis by placing them in 70% EtOH with 0.02% DEPC-treated water (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Histological studies

For tissue analysis, each uterus was surgically resected from the section where the fetal villi section of the caruncle and trophoblast cells/glandular epithelial cell section of the endometrium were visually identified. Subsequently, all uterine tissues were fixed in 70% DEPC (diethyl pyrocarbonate) ethanol for 24 h, dehydrated, made transparent in 95%-100% EtOH and xylene solution (Sigma, St. Louis, MO, USA) sequentially, embedded in paraffin, and stored. Before the experiment, paraffin blocks were deparaffinized in xylene solution after preparing tissue section slides with a thickness suitable for each experiment. Thereafter, they were sequentially treated in 95%-100% ethanol twice for 10 min and then in DW for 5 min and used in the experiment.

Representative sections of all uterus tissue paraffin blocks were randomly selected and sectioned at 10 µm thickness for use. They were subsequently stained with hematoxylin and eosin (H & E; Sigma-Aldrich, St. Louis, MO, USA). The stained slides were dehydrated, fixed with Permount solution (Fisher, NJ, USA), covered with cover glass, and histologically examined using an optical microscope (AX70, Olympus, Tokyo, Japan) (Kim *et al.*, 2018).

To investigate the occurrence of possible calcium deposition in the endometrium and caruncle region, 5

μm sections of paraffin-fixed tissue were incubated for 3 min with alizarin red staining. The interaction of alizarin red with calcium produces an orange/red product that is visible under a microscope (Oliveira *et al.*, 2013).

In-situ zymography

For the in situ zymography experiment, paraffin tissue was cut into 10 μm sections, hydrated, and boiled in 10 mM sodium citrate for 10 min. Then, it was cooled on ice for 20 min, washed three times for 5 min each with DW, and the emulsion (DW, 10% SDS, 2% Glycerol) and zymography reaction buffer (1M Tris-HCL, 5M NaCl, 1M CaCl₂, 0.2mM ZnCl₂, 0.2mM ZnCl₂, 0.2% Triton X-100, 0.02% NaN₃ in 1 \times phosphate-buffered saline (PBS); pH 7.5) was mixed at a ratio of 1:2 and mounted on slides. The slides were then placed in a box filled with 1M Tris and incubated at 37°C for 48 h. Routine H&E staining was performed for histological examination using an optical microscope (Kim and Yoon, 2021a).

Immunohistochemistry

For the immunohistochemical detection of IGF1-r, PAPP-A, and TIMP-2, paraffin tissue was sectioned at 5 μm and hydrated. Subsequently, antigen retrieval was performed by heating the sections in 10 mM sodium citrate (pH 6.0) at 95 °C for 5 min. For antigen unmasking treatment, the cells were treated with 0.3% hydrogen peroxide for 5 min at room temperature. Subsequently, the slides were subjected to antigen-blocking treatment with blocking buffer (3% bovine serum albumin diluted in 1XPBS) for 1h at room temperature. Insulin-like growth factor1 receptor (IGF1-r) (ab263903; Abcam, Cambridge, UK), pregnancy-associated plasma protein A (PAPP-A) (ab249813; Abcam), and tissue inhibitors of metalloproteinase 2 (TIMP-2) (ab230511; Abcam) were mixed in blocking buffer on the treated sample slide and incubated at room temperature for 2 h. Slides were washed in 1X PBS and incubated with HRP anti-rabbit IgG secondary antibody (ab288151; Abcam) or HRP Anti-Mouse IgG secondary antibody (ab47827; Abcam) for 1h at RT. After incubation, the sections were washed and activated for 10 min using the ABC detection kit (Vector, CA, USA). Diaminobenzidine (Vector, CA, USA) was used as the substrate for horse-radish peroxidase (HRP). The sections were stained with periodic acid-Schiff (PAS) reagent and hematoxylin solution containing 4% acetic acid. Tissues were dehydrated, removed, covered with Permount solution (Fisher Scientific, New Jersey, USA), and analyzed using light microscopy.

Immunofluorescence

This experiment was conducted using the appropriate

antibody dosage (10 $\mu\text{g}/\text{mL}$) and the test method provided by Abcam (Cambridge, UK). Paraffin blocks of uterine tissue from each group were sectioned to a thickness of 5 μm . Tissue slides were prepared following deparaffinization, hydration using xylene and ethanol, and permeabilization at -20°C with 0.1% Triton X-100 in 1 \times PBS (PBS-T). The samples were blocked at room temperature (RT) for 30 min in TPBS (1 \times PBS with 0.01% Tween-20) containing 5% normal horse serum (NHS) and 1% normal goat serum (NGS). All tissue slides were incubated in a dark room at RT for 1 h with primary antibody vascular endothelial growth factor (VEGF) (ab32152; Abcam) diluted 1:200 in blocking solution. To induce secondary anti-body conjugation, the cells were incubated in the dark with a blocking solution for 30 min. Subsequently, all slides were incubated with Alexa 594-conjugated anti-rabbit secondary antibody (ab150080; Abcam, Cambridge, UK) diluted 1:300 in blocking solution in a dark room at RT for 1 h. The secondary antibody solution was decanted and washed three times with PBS for 5 min each in the dark.

In-situ apoptosis detection

Fragmented nuclear DNA of apoptotic cells was detected by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) using an in-situ apoptosis detection kit (Takara, Shiga, Japan). Tissue slides were rinsed three times with 0.1% Triton X-100 in 1Xphosphate-buffered saline (PBST). Tissue slides were incubated with an end-labeling solution containing terminal deoxynucleotide transferase and anti-fluorescein-dUTP for 90 min at 37°C. After washing three times with PBST, the samples were incubated with peroxidase-labeled anti-fluorescein antibodies overnight at 4°C. All tissue slides were incubated in 0.1 $\mu\text{g}/\text{mL}$ DAPI for 1 min and rinsed with PBS. The coverslips were mounted with a drop of mounting medium. The slides were mounted using a fluorescent mounting medium (Dako, Carpinteria, CA, USA). Images of the detected proteins were analyzed using a fluorescence microscope (Olympus AX70). The Image J software (ver. 1.53t; National Institutes of Health, USA) was used to compare and analyze the detection level of the target protein by immunofluorescence analysis.

RESULTS

Uterine morphology and Ca²⁺ during pregnancy

As a result of evaluating the morphological difference in the uterus between water deer and sika deer (Fig. 1), we observed a difference in the overall size of the uterus and the distribution of each tissue type. In the case of sika deer, the myometrium was more widely distributed than in water deer, and the size of the villus cytotrophoblast zone

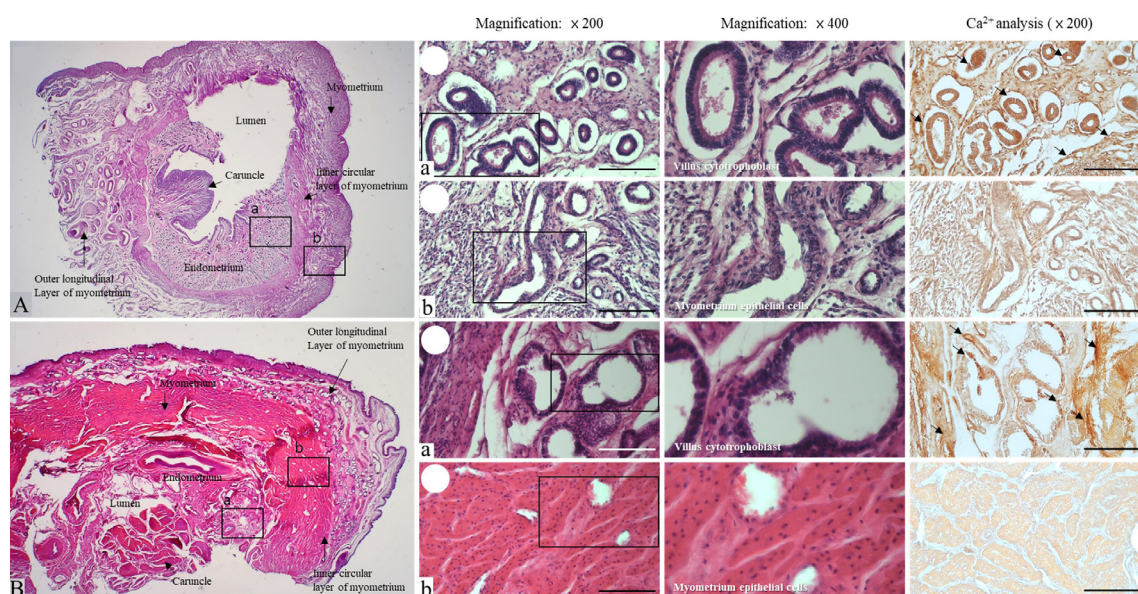


Fig. 1. Morphological analysis of endometrium during estrus and pregnancy in water deer and sika deer. This figure shows the overall morphological differences between water deer and sika deer uteri by H&E staining analysis. Water deer and sika deer exhibit differences in the shape of the uterus and the development of the endometrium. Detection of Ca^{2+} was concentrated in the endometrium and activated in the villus cytotrophoblast in both the water deer and the sika deer. Magnification = 20 \times .

in the endometrium was relatively large. In particular, the caruncle of water deer has a different shape than that of sika deer, and the shape of the fetal mesenchyme section and the distribution of the villi section differ. Regarding the distribution of the myometrium, the inner circular layer of the myometrium was clear in water deer, but there was no clear boundary in sika deer, indicating differences in muscle development. In the evaluation of Ca^{2+} deposition, water deer showed a very high deposition rate in the villus cytotrophoblast cell layer, which was considered to be more active than in sika deer. In contrast, the myometrium showed very low or unobservable Ca^{2+} deposition rates in both species.

Matrix metalloproteinases (MMPs) in the endometrium during pregnancy and expression patterns of growth-related proteins

Comparison of the expression patterns of genes (IGF1, PAPP-A) related to uterine growth in water deer and sika deer revealed that all proteins were expressed in the stroma cell sections of water deer (Fig. 2). However, the expression in the villus section was very low compared to that in sika deer. In particular, the expression of IGF1 and PAPP-A in sika deer was increased in the main BNCs of villus cytotrophoblasts and showed relatively high expression compared to that in water deer. In the myometrium, it is expressed in the stroma cell section and

exhibits higher expression in water deer than in sika deer. To measure morphological changes in the endometrium of the uterus, the activities of MMPs that promote changes in the extracellular matrix (ECM) were compared. The results showed very high activity in water deer compared to that in sika deer. The expression of TIMP2, which regulates MMPs, was relatively higher in the villus cytotrophoblast zone of sika deer than in that of water deer.

Detection of VEGF protein in the gestational endometrium

Analysis of the expression pattern of VEGF protein affecting angiogenesis revealed that VEGF expression was predominantly in the villus cytotrophoblast zone of the endometrium (Fig. 3). In particular, water deer showed increased VEGF expression in all parts of the uterus. In sika deer, expression was confirmed within villous cytotrophoblast cells like water deer; however, it was expressed in the section where each BNC developed rather than the overall section of tissue. It was mostly detected in the endometrial villus cytotrophoblast zone and myometrium extravascular zone. In the case of sika deer, high detection was confirmed in the BNCs of the gland zone of the endometrium. The overall expression pattern was higher in the BNCs of sika deer than in those of water deer. The overall difference was that VEGF detection in water deer was higher in the endometrium than that in sika deer.

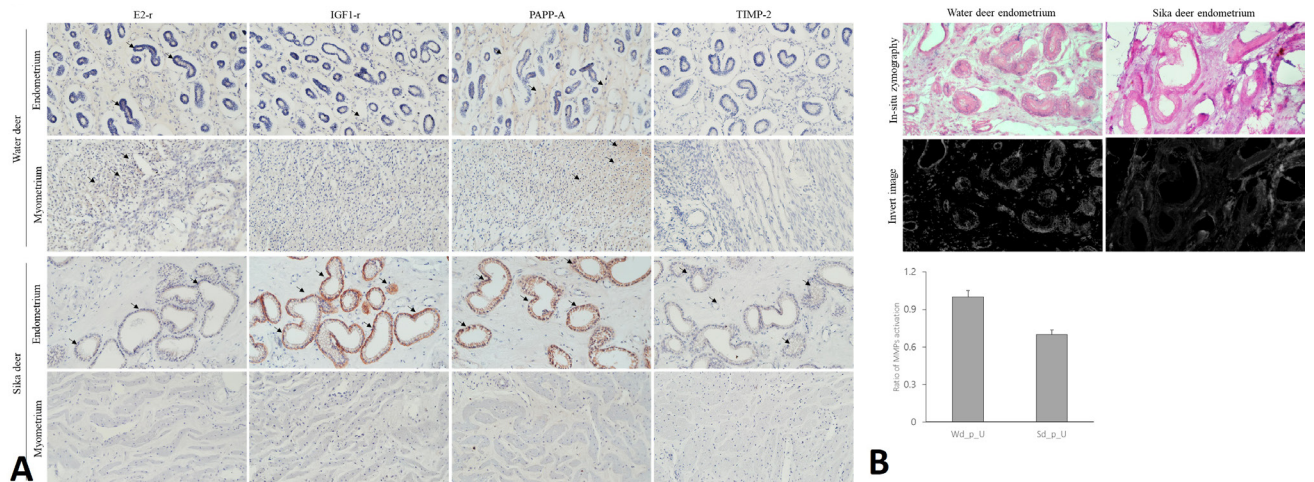


Fig. 2. Tissue immunohistochemistry and activity assay of MMPs. A: Analysis of protein expression in endometrial tissue, B: Analysis of the activity of MMPs in endometrial tissue (in-situ zymography). In the tissue immunological analysis, water deer generally expressed MMPs in stromal cells, whereas sika deer mainly expressed MMPs in villus cytotrophoblasts. MMPs were active in all parts of the endometrium in both water deer and sika deer. Black arrows indicate areas with distinct expressions. Magnification = 200 \times . In-situ zymography data were analyzed using Welch's t-test, fold change, and GLM in SAS (ver 9.4; Statistical Analysis System Institute, Cary, NC, USA). All data are presented as mean \pm SEM. *Different letters within the same column represent significant differences between groups ($p < 0.05$).

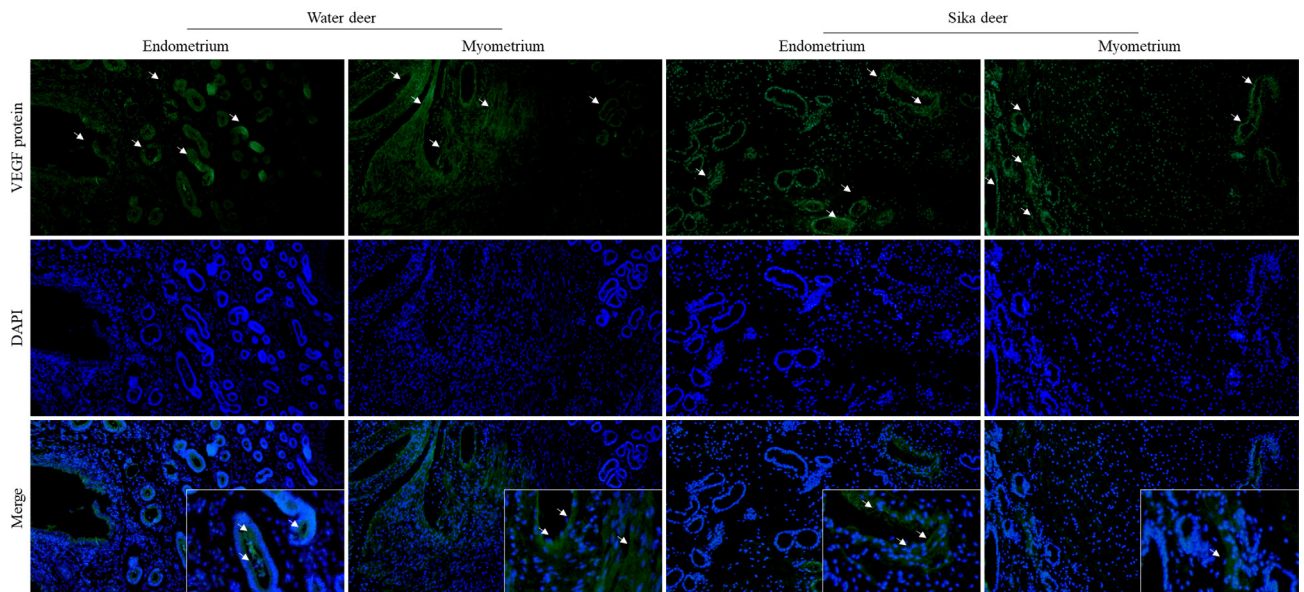


Fig. 3. Immunofluorescence detection of VEGF protein expression patterns in the uterus of early pregnancy. Expression in water deer was predominantly in stromal cells of the tissue body; in sika deer, expression was in the BNCs of the villus cytotrophoblasts. White arrows indicate areas with distinct expressions. Green fluorescence indicates gene expression. Magnification = 200 \times .

Detection of apoptosis in the endometrium during pregnancy

As a result of confirming the apoptotic activity in the endometrium to analyze the difference in maintenance and reconstitution of the uterus (Fig. 4), the apoptotic activity in the BNC section of the overall villus cytotrophoblast zone

of sika deer was relatively higher than that of water deer. In particular, the apoptotic activity of both water deer and sika deer was high in the stromal cells connecting the tissue surrounding the fetal villus zone. However, the activity was relatively higher in sika deer than in water deer.

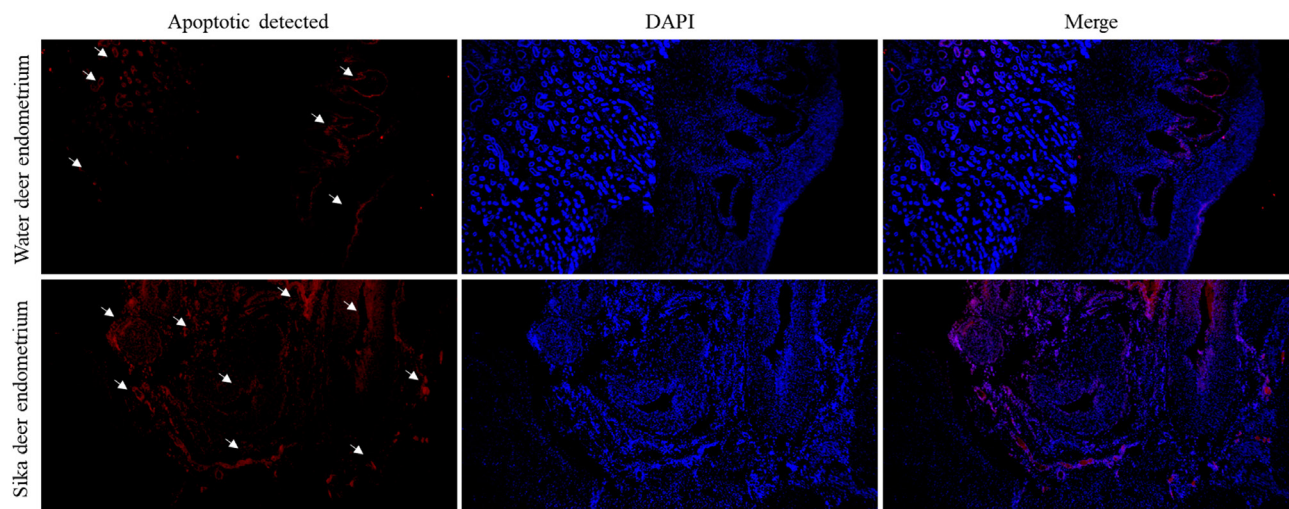


Fig. 4. Detection of apoptosis in endometrial tissue of water deer and sika deer. In the apoptotic detection assay, terminal deoxynucleotidyl transferase was used to label the 3-OH terminus of DNA fragments generated during apoptosis. White arrows indicate areas with distinct expressions. Magnification = $\times 200$.

DISCUSSION

The fusion between trophoblast cells and uterine endometrial cells of the embryo affects fetal development and enhances the maintenance of pregnancy through the formation of the cervical placenta and the signaling system with the embryo during early pregnancy (Kim *et al.*, 2018; Li *et al.*, 2019; Northrop-Albrecht *et al.*, 2021).

The early uterus is connected to the ovary during estrus through signal transduction. In particular, progesterone (P4) secreted by the corpus luteum in the ovary plays an important physiological role under the control of pituitary hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)), affecting the release of mature oocytes, supporting embryo implantation, suppressing myometrial contraction during pregnancy, and entering the maintenance phase to prepare for pregnancy. At the onset of pregnancy, the uterus undergoes drastic changes (Kim *et al.*, 2014; Li *et al.*, 2019). After successful implantation of the fertilized egg, the uterus again undergoes gross tissue changes. At this stage, the placenta produces steroids in most mammalian species, such as cattle (Shemesh, 1990), pigs (Meyer, 1994), roe deer (Naidansuren *et al.*, 2012), and humans (Tuckey, 2005), and structural changes occur in the uterus.

In our study, the development of the villus cytotrophoblast zone in the endometrium of sika deer was greatly increased compared to that of water deer. However, the Ca^{2+} deposition rate increased in the villus cytotrophoblasts of the water deer, suggesting that changes in the uterus between the two species reacted differently. In

particular, progesterone levels in pregnant sika deer varied individually, but as the gestation period increased, fecal progesterone increased significantly enough to be detected, and it is possible that the composition of the uterus might have changed (Spencer *et al.*, 2005; Kotlarczyk *et al.*, 2021). In particular, the activities of PAPP-A and IGF1 were increased in the villus cytotrophoblasts of sika deer. In addition, the activity of MMPs, which promote morphological changes in the ECM constituting the endometrium, is thought to cause overall tissue changes by acting in the villus cytotrophoblast zone (Kim *et al.*, 2014, 2018, 2021b). However, compared to sika deer, water deer showed fewer changes in the endometrium, and the activity of MMPs was also relatively low.

In general, the physiological effects of P4 show a clear difference between water deer and sika deer. In particular, VEGF, which plays an essential role in tissue remodeling, is rapidly activated in the uterus and promotes the proliferation of vascular epithelial cells around the tissue. Thereafter, it is presumed to form new cytokines, induce apoptosis in the surrounding tissues, and promote tissue remodeling (Han and Jiang, 2008; Huang *et al.*, 2013; Gonçalves *et al.*, 2015). In our study, VEGF was increased in the villus cytotrophoblast zone and extravascular zone of the myometrium in water deer and sika deer, and it seems that apoptosis was also induced accordingly (Huang *et al.*, 2013; Zhao *et al.*, 2008). In particular, the apoptotic action in the stroma cells connecting the tissue section around the fetal villus zone appears to be similar to the activity of VEGF. Therefore, VEGF activity and apoptosis are related, and reduced VEGF secretion from normal endometrial

stromal cells (ESC) in the uterus appears to stabilize the tissue (Gonçalves *et al.*, 2015; Kim *et al.*, 2021b). Unlike previous studies, changes in the uterus during pregnancy in water deer and sika deer are simultaneously formed by the apoptosis of BNCs, the action of VEGF, and tissue reorganization by maximizing changes in endometrial cells. It is thought that this is accelerating (Asher *et al.*, 1999; Kim *et al.*, 2014; Kotlarczyk *et al.*, 2021).

The results of this study show that water deer and sika deer differ greatly in maintenance and change of the uterus during pregnancy, and among animals with the same silent ovulations, the change in the uterus of the sika deer differed drastically from our expectations. Undoubtedly, morphological differences in the uterus cannot explain everything regarding pregnancy maintenance and fetal development in sika deer, but, in general, the pregnancy maintenance action of sika deer is significantly different from that of water deer in the same wild state. During this period, it can be assumed that embryo implantation and fetal development may occur more slowly than in other ungulates.

CONCLUSION

Our study aimed to determine differences in reproductive physiology through tissue reconstruction of the gestational uterus of wild water deer and sika deer. The results showed a notable difference in the changes in the villus cytotrophoblast zone of the endometrium and the extravascular zone of the myometrium, which are involved in the development of early gestation in sika deer and water deer. It was confirmed that endometrial remodeling in sika deer was more rapid than that in water deer by the activity of MMPs and apoptotic action. Therefore, there is a measurable difference in functional changes between water deer and sika deer. Compared to water deer, it is believed that sika deer exhibit a difference in pregnancy maintenance by forming rapid changes in the uterus during fetal development. Accordingly, this study contributes basic data on the reproductive physiological conditions for the Korean restoration of nationally extinct sika deer.

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IRB approval

The Institutional Animal Management and Use

Committee of the Wildlife Conservation and Research Center (Capture Permit No. 2013-1).

Ethical statement

The study protocol was approved by the Committee on the Ethics of Animal Experiments of Hankyong National University, South Korea (IACUC approval HK-2022-1). All experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in Korea.

Statement of conflict of interest

The authors have declared no conflict of interest.

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