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Expression of Estrogen Receptor-α like Immunoreactivity in the Endometrium and Superoxide Dismutase Activity after Addition of Estrogen in Culture Medium of Endometrial Epithelial Cells in the Rabbit

[1]Miho Wada, [2]Makoto Yokosuka, [2]Tomoaki Nakada, [1]Masanori Kobayashi,
[1]Tatsuya Hori and [1]Eiichi Kawakami
[1]Laboratory of Reproduction,
[2]Laboratory of Comparative and Behavior Medicine,
Nippon Veterinery and Life Science University, 1-7-1 Kyounan-Cho,
Musashino, 180-8602 Tokyo, Japan

Abstract: The purpose of this study is to reveal the effect of estrogen-inducing SOD activity on the endometrium of domestic rabbits. Researchers measured Superoxide Dismutase (SOD) activity to investigate the reason of occurrence of popular uterine adenocarcinoma in rabbits in estradiol-17β-stimulated endometrium epithelial cell culture. Then, we also examined the distribution of cells expressing Estrogen Receptor α (ERα)-like Immunoreactivites (ERα-like-IR) in the uterus prone to developing adenocarcinoma. The addition of estradiol-17β to the primary culture of endometrial epithelial cells obviously decreased SOD activity. Moreover, the areas of endometrium in which uterine glands were confined in control rabbits and notably larger in rabbits with glandular hyperplasia of the uterus compared in normal dog uterus that the occurrence of uterine adenocarcinoma is rare. ERα-like-IR cells were found in all proliferating glands in rabbits with hyperplasia.

Key words: Endometrial epithelial cell, estrogen receptor α, rabbit, superoxide dismutase, uterine adenocarcinoma

INTRODUCTION

In domestic rabbits, the incidence of uterine adenocarcinoma increases after age 4 years (Heatley and Smith, 2004) and uterine adenocarcinoma is thought to arise from endometrial hyperplasia (Saito *et al*., 2002). Although, rabbits are continuous breeders in the case of the pet-rabbits rarely have the chance to conceive and are thus continuously exposed to high levels of estrogen released from the ovary. It is likely that resulting endometrial hyperplasia increases the risk of uterine cancer in these rabbits. Superoxide Dismutases (SOD) are natural antioxidant enzymes that play a role in removing excess Reactive Oxygen Species (ROS) (Bannister *et al*., 1987). Accumulation of ROS is known to damage to DNA and carcinogen in humans (Nakabeppu *et al*., 2006; Valko *et al*., 2004). It has been reported that low SOD activities in human prostate (Mikhak *et al*., 2008) and canine testis (Kawakami *et al*., 2007b) are a factor of occurrence of some tumors. These previous findings suggest that decreases in SOD activity as a consequence of elevated sensitivity to estrogen in the endometrium plays a role in inducing uterine cancer in rabbits. Researchers had a hypothesis that the endometrial estrogenic sensitivity was involved in estrogen-inducing SOD activity.

Thus in this study, researchers investigated the effect of an estrogenic agent on the endometrium, induction of SOD and distribution of Estrogen Receptor α-like-Immunoreactive (ERα-like-IR) in the endometrium of domestic rabbits. Moreover as the occurrence of uterine adenocarcinoma in the dog is very few (Pires *et al*., 2010), we also examined the distribution of estrogen receptor containing cells in the endometrium of rabbits was compared with it of dogs.

MATERIALS AND METHODS

All the uterine tissues used for this study were obtained during routine ovariohysterectomy under general anesthesia at the animal hospital at Chiba-prefecture, Japan. Before inclusion the owner signed an informed consent to participate. Three uteri-sample from healthy (normal) 7 months to 2 years old rabbits, two uteri-sample from rabbits with glandular hyperplasia of the uterus (3-4 years old) and two-uterine

Corresponding Author: Eiichi Kawakami, Laboratory of Reproduction, Nippon Veterinery and Life Science University, 1-7-1 Kyounan-Cho, Musashino, 180-8602 Tokyo, Japan

horns removed by contraceptive operations from normal dogs were used for the ERα-like immunohistochemical study. All of the rabbits and dogs had developed follicles in the ovaries and were estrous period. Fresh endometrial epithelial cells were collected from three healthy rabbits uteri and cultured in the medium with and without supplementation of estradiol-17β to examine changes in SOD activity induced by estradiol-17β.

Coronal samples (5 mm thickness) of the uterine horns were prepared and immediately immersed in fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer; PB, pH 7.4). After incubation at 4°C for 48 h, the fixed uterine samples were stored in 30% sucrose in 0.1 M PB (pH 7.4) at 4°C until use. Using a cryostat (Leica, USA) serial coronal sections of the uterine horn (15 μm thick) were prepared from sucrose-treated uterine samples for histochemical examination. Sections were mounted on poly-L-lysine coated glass slides (Matunami, Japan). Immunocytochemistry performed as described previously (Yokosuka and Hayashi, 1992; Yokosuka *et al*., 1997).

For the culture experiments, uteri were removed from three rabbits under inhalation anesthesia and placed immediately in saline. Endometrial epithelial cells were recovered from these uteri for cell culture according to the method described by the previous report (Takahashi *et al*., 2001). Each uterine horn was cut vertically and the uterine lumen was exposed. The endometrial epithelium was scraped off using a surgical blade and incubated for 5 min in 5 mL Eagle's Minimum Essential Medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 38°C. The cell suspension was passed through a nylon wool column (70 μm; Becton Dickinson, Franklin Lakes, NJ) and centrifuged for 10 min at 100 g. The resulting pellet, containing erythrocytes and epithelial cells was then recovered and subjected to density gradient centrifugation for further purification. The pellet was resuspended in 1 mL of fresh MEM. The number of endometrial epithelial cells was counted using a hemocytometer. Next, 20 ng of estradiol-17β (Sigma Chemical Co.) with penicillin (100 IU mL^{-1}, Sigma Chemical Co.) and streptomycin (100 μg mL^{-1}, Sigma Chemical Co.) were added to each well. A culture medium without estradiol-17β was prepared as a control. Epithelial cells were plated at 0.9 mL volumes into wells of 4-well tissue culture dishes (Nunc Co. Ltd., City, Denmark) and cultured in the same culture medium under an atmosphere of 5% CO_2 in air at 38°C. The culture medium was changed every 2-3 days. After culturing for 7 days, SOD activity levels in culture media recovered from the estradiol-17-stimulated and control cultures were measured and compared. The SOD activity in the culture media was measured by enzyme analysis reactions using a SOD Assay kit (Trevigen, Inc., MD, USA) and a spectrophotometer with absorbance at 550 nm. Total protein concentration in the culture media was determined by the method of Bradford (Kawakami *et al*., 2007a).

RESULTS

In normal rabbits, uterine glands which containing ERα like Immunoreactivity (ERα-like-IR) cells were only restricted at outer layer of the endometrium (Fig. 1a and b). This ERα-like-IR revealed that the relative endometrial areas wherein uterine glands were distributed were smaller in normal rabbits while being distributed across the endometrium in dogs (Fig. 1c and d). On the other hand in rabbits with endometrial hyperplasia, proliferation of uterine glands was enhanced along with thickening of the endometrium and ERα-like-IR cells were found in all excessively proliferating glands (Fig. 1e and f).

On the other after the 7 day culture of endometrial epithelial cells recovered from control rabbits, the mean SOD activity in the media was 1.42 units mg^{-1} protein in the control group and notably lower at 0.65 unit mg^{-1} protein in the estradiol-17β-stimulated group (Table 1).

The findings suggested as follows. The result of the distribution pattern of the ERα-like-IR which we examined in the normal and in the endometrial hyperplasia-rabbit uterine glands in this study were extremely resemble the results of previous reports (Asakawa *et al*., 2008; Parillo *et al*., 2013) therefore, researchers believe that the results of immunohistochemical staining of this study were reflect of the fact the change of the ERα of the rabbit uterine glands. In rabbits, these endometrial hyperplasia with the increase of the ERα containing cells will be results in elevated uterine sensitivity to endocrine estrogens. Although, rabbits are continuous breeders, the pet-rabbit rarely have the chance to conceive and consequently. Thus, they are continuously exposed to high levels of estrogen released from the ovary. The elevated formation of ERα-like-IR expressing uterine glands associated with ageing-associated endometrial hyperplasia will be results in elevated uterine sensitivity to endocrine estrogens in pre-uterine carcinoma state rabbits. The SOD production and activity in the endometrium of rabbits may decrease by the effect of

Table 1: Superoxide dismutase activity (unit/mg protein) after addition of estradiol-17β in culture medium of endometrial epithelial cells from 3 rabbits

Parameters	No. 1	No. 2	No. 3	Mean
Control	0.81	2.05	1.41	1.42
Estradiol	0.33	0.61	1.01	0.65

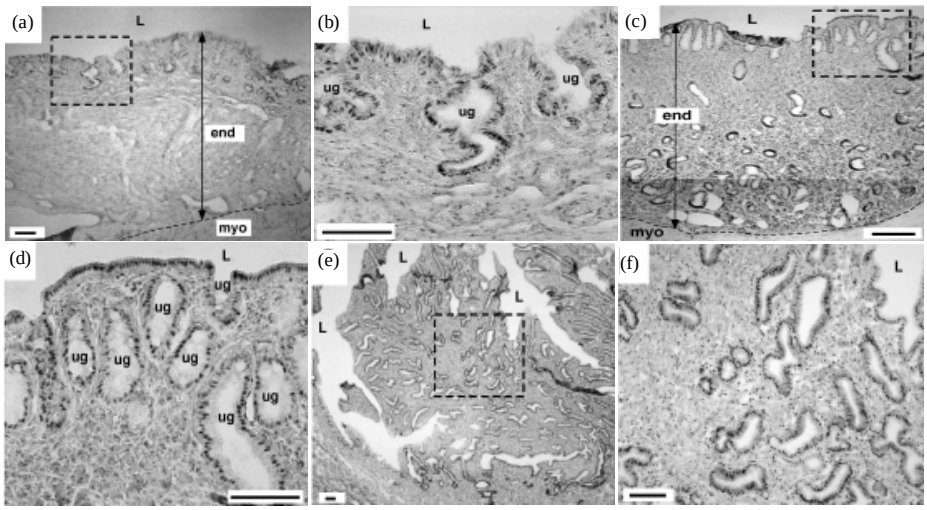

Fig. 1: ERα-like-IR in the endometrial uterine gland in the rabbit (a-f) and dog (c-d). In normal rabbit (a-b), ERα-like-IR containing uterine glands are distributed on the outer layer of the endometrium only. In control dogs (c-d), ERa-like-IR containing uterine glands are distributed across the whole lining membrane of the endometrium. In the case of endometrial hyperplasia rabbit (e-f) ERα-like-IR containing uterine glands are present across the whole endometrium but are concentrated in the outer layer b, f and d show the respective square areas in a, e and c at a higher magnification. L: Lumen of the uterus; end: endometrium, myo: myometrium; ug: uterine gland; Scale bars = 100 μm

estrogen continuously secreted from the ovaries of rabbits. The consequent decline in SOD activity within the endometrium induces the increase of ROS levels and may become one of risk factors of occurrence of uterine adenocarcinoma in the rabbit.

CONCLUSION

These results suggest that the low SOD activity by the effect of estradiol-17β in the rabbit uterus may be one of risk factors of occurrence of uterine adenocarcinoma.

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