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Original Article

Influence of the estrus cycle on the evaluation of a vaginal irritation study in intact and ovariectomized rats

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Abstract: When conducting vaginal irritation studies, ovariectomized rats or rabbits are typically used according to practical reports. In the present study, we evaluated the influence of the estrus cycle in a vaginal irritation study using intact rats and ovariectomized rats, which exhibit a late diestrus-like condition, to determine whether intact rats can be useful for evaluating vaginal irritancy. Rats were divided into 4 groups: proestrus, estrus, and metestrus or diestrus in intact rats and ovariectomized rats. All the rats in each group were treated with a vehicle or sodium dodecyl sulfate, as the irritant, in single-dose and 4-day repeat-dose vaginal irritation studies. Each rat's vagina was examined histopathologically, and the irritation score was calculated using a semiquantitative scoring system. In the single-dose study, the irritation scores for the proestrus or ovariectomized groups treated with sodium dodecyl sulfate were higher than those of the estrus group or metestrus or diestrus group. In the 4-day repeat-dose study, a significant histopathological difference was not found among the intact rats (proestrus, estrus, and metestrus or diestrus groups), and the irritation score range of the intact rats was similar to that of the ovariectomized rats, though the mean score of the intact rats was slightly lower than that of the ovariectomized rats. These results suggest that intact rats might be well suited for 4-day vaginal irritation studies and useful for evaluating vaginal irritancy using not only the mean score, but also individual irritation score ranges, whereas the estrus cycle would need to be identified in single-dose vaginal irritation studies. (DOI: 10.1293/tox.2016-0059; J Toxicol Pathol 2017; 30: 161–168)

Keywords: vaginal irritation study, estrus cycle, intact rats, ovariectomized rats, sodium dodecyl sulfate

Introduction

Vaginal irritation studies are conducted as nonclinical safety assessments to determine the vaginal irritation potential of newly developed compounds intended for vaginal application. According to practical reports[1, 2], rabbits or ovariectomized (OVX) rats are typically used when nonclinical vaginal irritation studies are conducted. In addition, several animal species including monkeys, pigs, and dogs have been used to assess vaginal irritancy, but these animals are not considered to be appropriate for screening studies because of ethical issues, cost, or handling[3].

Rabbits are currently the standard species used for initial evaluations of vaginal irritation potential. However, rabbits may be too sensitive for the evaluation of vaginal irritation, since the vaginal mucosa of rabbits consists of a single columnar cell layer. This morphological feature of the vaginal mucosa in rabbits may induce overestimation in evaluation of the irritancy of compounds when compared with the vaginal mucosa of humans, which consists of a thick, non-keratinizing, stratified, and squamous epithelium. Kaminsky *et al.* demonstrated that the sensitivity of the vaginal mucosa to changes in the pH of a liquid cosmetic douche formulation was greater in rabbits than in rats[4], and they considered that the human vagina might be less sensitive and that the response in humans might more closely resemble that in rats. They also demonstrated that the vagina of rabbits was clearly more sensitive to nonoxynol-9 than that of rats, and marked epithelial exfoliation, submucosal edema, and inflammatory cell infiltration were observed in histopathological examinations of the vaginas of rabbits[5]. Based on the abovementioned information, rabbits are expected to provide a more exaggerated representation of a compound's irritation potential in the human vagina. Therefore, rats might be useful for evaluating vaginal irritancy so as not to overestimate the toxicological risk when evaluating candidate compounds.

On the other hand, the vaginal tissue of intact rats might not be suitable for evaluating vaginal irritancy in view of their relatively short estrus cycle and their cornified vaginal mucosa during the estrus phase[6]. The cornified vaginal mucosa of intact rats is expected to be less permeable and more resistant to damage from vaginal irritancy. To overcome

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these concerns, the use of OVX rats, which exhibit a late diestrus-like condition characterized by a uniform, thin, and noncornified vaginal epithelium[7], has been recommended for evaluations of vaginal irritation potential[1]. However, differences in irritancy between intact rats and OVX rats have not been reported. In addition, the influence of the estrus cycle in intact rats when evaluating vaginal irritancy has not been previously examined. Considering that an ovariectomy requires more time to prepare the test subjects and causes pain to the rats, we felt that the relative inconvenience of using OVX rats should be reevaluated.

The aim of the present study was to evaluate the influence of the estrus cycle in intact rats compared with OVX rats and to determine whether intact rats can be useful for evaluating vaginal irritancy. We performed single-dose and 4-day repeat-dose vaginal irritation studies and compared the irritancy experienced by intact rats evaluated according to the estrus cycle and OVX rats.

Materials and Methods

Experimental protocol

Animals: Sprague-Dawley rats were obtained from Charles River Laboratories Japan (Yokohama, Japan) and were acclimated until use. The rats were maintained using a 12-hour light/12-hour dark cycle, a constant temperature of 23 ± 3°C, and a relative humidity of 50 ± 20%. Animal care and use conformed with the guidelines for the Institutional Animal Care and Use Committee of Taisho Pharmaceutical Co., Ltd. Seventy-two female rats, 4 weeks of age, were prepared. At 6 weeks of age, the rats were divided into 2 groups: OVX group and non-OVX intact group.

Ovariectomy: In the OVX group, a bilateral ovariectomy was performed at 6 weeks of age. The ovariectomy was performed under isoflurane anesthesia by placing the animal in a dorsal position in accordance with Steel *et al.*[8].

Estrus cycle determination and grouping: Estrus cycles were determined using vaginal smears performed during the early morning on the first day of administration at 12 weeks of age. Vaginal secretions were collected and then stained with 5% Giemsa Stain Solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The characterization of each phase of the estrus cycle was based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cells, and leukocytes. The stages of the estrus cycle were identified by examining the vaginal smears, and the rats were divided into 3 groups: proestrus (PE), estrus (E), and metestrus or diestrus (ME-DE).

In the OVX group, a daily vaginal smear was examined at 10 weeks of age, i.e., at 27 days to 31 days after ovariectomy, to confirm that complete OVX had been performed.

Vaginal irritation study: For the test material, sodium dodecyl sulfate (SDS, code no. 196-08675, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was used. Vaseline (code no. 224-00165, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was used as the vehicle. At 12 weeks of age, each group (PE, E, ME-DE, or OVX) was treated with the vehicle or SDS (5% or 30%) in a single-dose (once) vaginal irritation study (3 animals per group) and with the vehicle or SDS (5% or 20%) in a repeat-dose (once daily, 4 days) vaginal irritation study (3 animals per group). The SDS concentration was set based on the results of a preliminary study. Briefly, slight histopathological changes were observed at a low dose (5% SDS), and high irritation scores were observed at high doses (20 or 30% SDS). At 30% SDS, histopathologically remarkable changes were noted even after a single administration. From these results, 30% and 20% were set as the highest doses for the single- and repeat-dose studies, respectively. SDS or the vehicle (0.2 mL) was introduced directly into the vagina using a disposable syringe and a sonde needle.

Histopathological evaluation

The rats were sacrificed by exsanguinations under isoflurane anesthesia 24 hours after the last administration, and the vagina, uterus, and ovaries (except in the OVX group) were examined macroscopically and removed. The vagina was opened longitudinally and was fixed in 10% neutral buffered formalin. Three levels of the vagina (low, middle, and upper segments), uterus, and ovaries were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The stained histology sections were examined using light microscopy. Three levels of the vagina were examined and graded using a scoring system[1, 2], as follows: epithelium score, 0 = intact-normal, 1 = cell degeneration or flattening of the epithelium, 2 = metaplasia, 3 = focal erosion, and 4 = generalized erosion or ulceration; leukocyte score, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked; edema score, 0 = absent, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked; and vascular injection score (congestion/hemorrhage), 0 = absent, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked with disruption of vessels. The average epithelium, leukocyte, edema, and vascular injection scores were then calculated by averaging the scores for the three levels, and the average scores were then summed to obtain the irritation score. The irritation score for each rat was rated as follows: 0 = none, 1 to 4 = minimal, 5 to 8 = mild, 9 to 11 = moderate; and 12 to 16 = marked irritation. Then, the score for each rat was averaged. The mean irritation score (MIS) and the acceptability ratings were as follows: 0 to 8 = acceptable, 9 to 10 = marginal, and 11 or greater = unacceptable. The uterus and ovaries were examined microscopically to determine of the estrus stage as supplementary information.

Results

Single-dose vaginal irritation study

Histologically, the vaginal estrus cycle at the time of necropsy (approximately 24 hours after treatment) was at the estrus stage in 6 of the 9 rats (Table 1, Fig. 1A) and the metestrus stage in 2 of the 9 rats in the PE group treated with Vaseline and SDS. In 1 of the 3 rats in the PE group treated with 30% SDS, the estrus stage could not be deter-

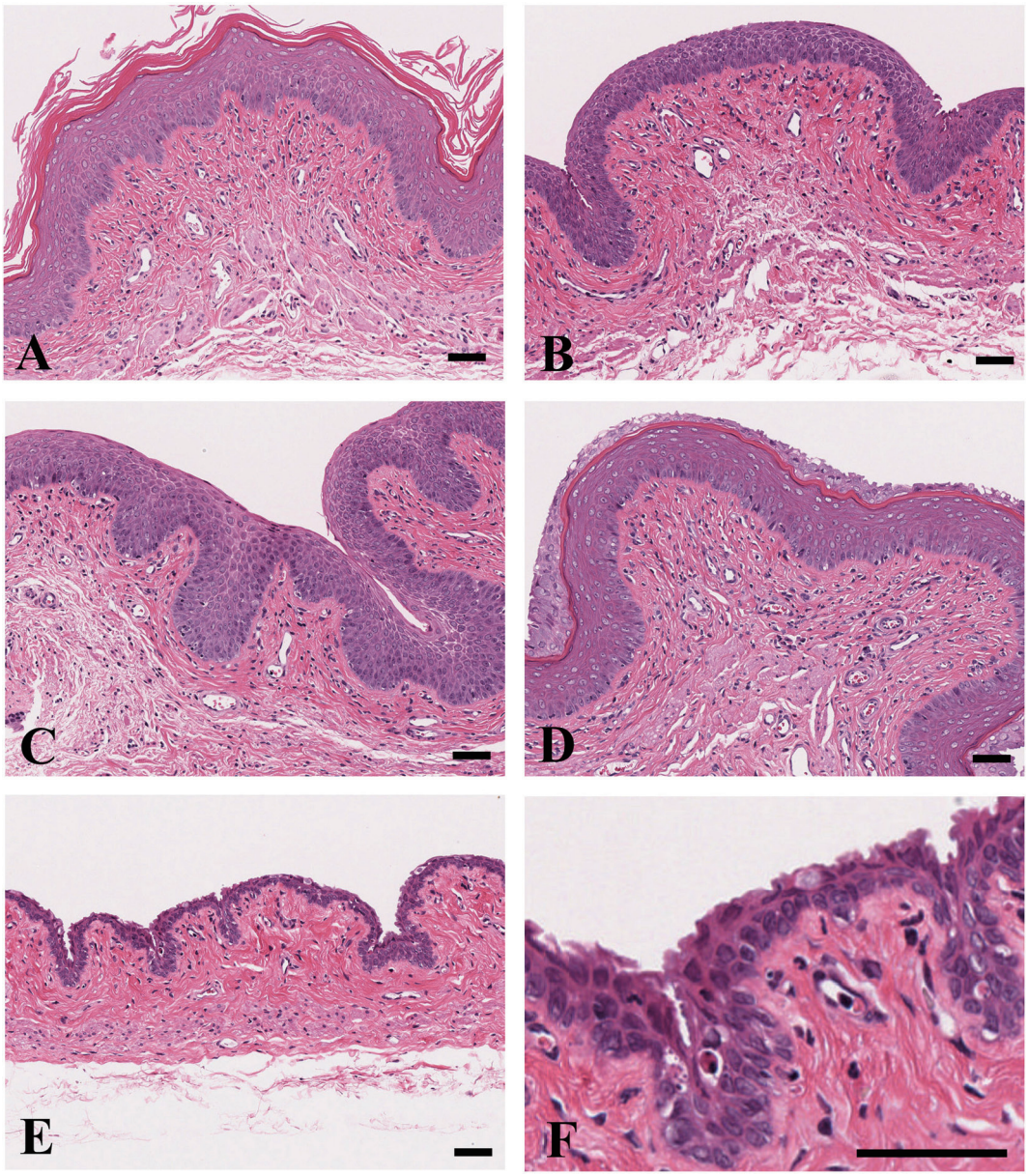

Fig. 1. Vaginal morphology at the time of necropsy at various stages of the estrus cycle in rats treated with a vehicle (Vaseline) in a single-dose vaginal irritation study. A: Estrus (in PE group). B: Metestrus (in E group). C: Diestrus (in ME-DE group). D: Proestrus (in ME-DE group). E: An ovariectomized rat (in OVX group) showed a markedly atrophic stratified epithelium with two or three cell layers and many degenerated epithelial cells. F: Higher magnification of E. H&E stain. Bars = 50 µm.

mined based on a vaginal examination, since most of the vaginal epithelium had been exfoliated; the uterus and ovaries, however, were in the estrus stage. In the E group, all the Vaseline and SDS-treated animals were histologically found to be in metestrus (Table 1, Fig. 1B). In the ME-DE group, 6 of the 9 Vaseline and SDS-treated animals were histologically found to be in diestrus (Table 1, Fig. 1C), whereas the other 3 animals were found to be in proestrus (Table 1, Fig. 1D).

In the OVX group, the ovariectomized animals exhibited an overall thinning of the vaginal wall (Fig. 1E); the epithelium of the vagina was thinner than that of the diestrus rats and showed a markedly atrophic stratified epithelium with two or three cell layers and many degenerated epithelial cells (Fig. 1F, epithelium score = 1).

Histopathologically, exfoliation of the vaginal epithelium, mild to severe inflammatory cell infiltration, and minimal to moderate edema of the muscular and adventitial layers were observed in an SDS dose-dependent manner in the PE group treated with SDS, (Fig. 2A). In the E or ME-DE groups treated with SDS, focal ulceration of the vaginal epithelium and mild to severe inflammatory cell infiltration were observed (Fig. 2B), though the areas of the epithelial lesions were smaller than those in the PE group treated with

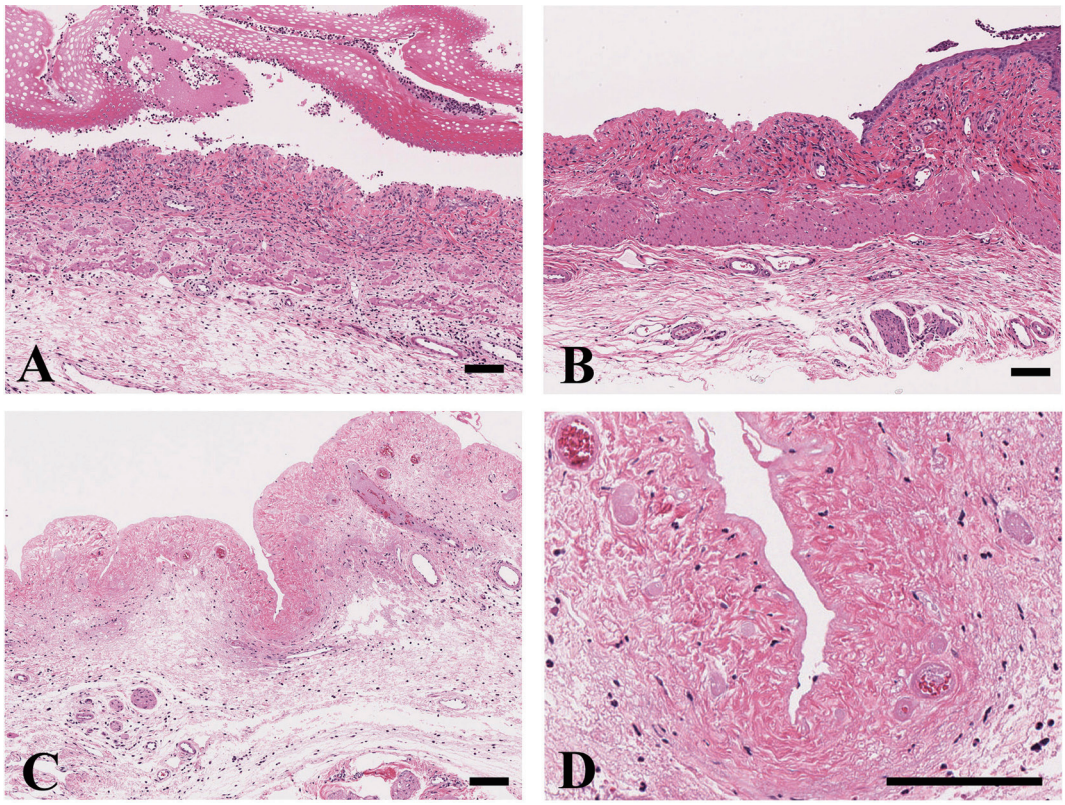

Fig. 2. Histopathological findings of the vagina in rats treated with 30% SDS in a single-dose vaginal irritation study. A: Exfoliation of the vaginal epithelium, severe inflammation, and edema of the muscular and adventitial layers are observed (scores: epithelium, 4; leukocytes, 4; edema, 3; vascular injection, 0; PE group). B: Focal ulceration of the vaginal mucosa and inflammatory cell infiltration are observed (scores: epithelium, 4; leukocytes, 3; edema, 0; vascular injection, 0; ME-DE group). C and D: Severe edema of the muscular and adventitial layers (C) and necrosis of the vessels in the lamina propria (D) are observed in addition to severe inflammation and ulceration of the vaginal mucosa (scores: epithelium, 4; leukocytes, 3; edema, 3; vascular injection, 2; OVX group). H&E stain. Bars = 100 μm.

SDS. In the OVX group treated with SDS, exfoliation of the vaginal epithelium, and mild to severe inflammatory cell infiltration were observed in an SDS dose-dependent manner. In addition, edema of the muscular and adventitial layers (Fig. 2C) and necrosis of the vessels of the lamina propria (Fig. 2D) were observed in the OVX group treated with 30% SDS.

In the scoring evaluation, the irritation score increased in an SDS dose-dependent manner in each group (PE, E, ME-DE, and OVX) (Table 1). The MIS after Vaseline treatment was 2 points in all the groups (PE, E, ME-DE, and OVX), those of the groups treated with 5% SDS were 8 (PE), 5 (E and ME-DE), and 7 (OVX), and those of the groups treated with 30% SDS were 9 (PE), 7 (E and ME-DE), and 11 (OVX).

In the PE group treated with SDS, the MISs for both doses were higher than that in the E or ME-DE groups treated with SDS. In most animals in the PE group treated with SDS, the high irritation score was influenced by the addition of the edema score, as edema was not scored in the E or ME-DE group treated with SDS. The epithelium scores in the E and ME-DE groups treated with 30% SDS were both 4 (highest score), though the areas of the epithelial lesions were smaller than those in the PE group treated with 30% SDS.

In the single-dose study, the highest MIS was observed in the OVX group treated with 30% SDS, and this was influenced by the addition of the vascular injection score, as vascular injection was not scored in the PE, E, or ME-DE groups.

The acceptability rating of each of the 5% SDS groups was “acceptable.” At 30% SDS, the acceptability ratings of the E and ME-DE groups were both “acceptable,” and those of the PE and OVX groups were “marginal” and “unacceptable,” respectively.

Repeat-dose vaginal irritation study

The stage of the histological vaginal estrus cycle at the time of necropsy on day 4 varied and was not correlated with the initial stage of the estrus cycle, which was used for selecting each PE, E, and ME-DE group at the time of first administration (Table 2).

Grossly, dark red foci were observed in the vagina in 2 of the 3 rats in the ME-DE group treated with 20% SDS, and a red focus was observed in the vagina in 1 of the 3 rats in the OVX group treated with 20% SDS. In these rats,

Table 1. Single-dose Vagina Irritation Study

Test article	Vaseline												SDS 5%												SDS 30%											
Group	PE			E			ME–DE			OVX			PE			E			ME–DE			OVX			PE			E			ME–DE			OVX		
Animal No.	11	12	13	21	22	23	31	32	33	41	42	43	51	52	53	61	62	63	71	72	73	81	82	83	91	92	93	101	102	103	111	112	113	121	122	123
Estrus cycle of vagina	ME	E	E	ME	ME	ME	PE	PE	DE	–	–	–	E	ME	E	ME	ME	ME	DE	DE	DE	–	–	–	E*	E	E	ME	ME	ME	DE	PE	DE	–	–	–
Findings																																				
Epithelium	1.0	0	0	1.0	0.7	1.0	0	0	0	1.0	1.0	1.0	4.0	2.7	3.3	2.3	3.0	1.0	1.3	2.7	2.3	3.0	4.0	4.0	4.0	4.0	4.0	3.7	3.0	3.7	4.0	3.7	3.7	4.0	4.0	4.0
Leukocytes	1.7	2.0	2.0	1.3	2.0	1.0	1.3	2.0	1.7	1.7	1.0	1.0	4.0	3.0	4.0	3.0	3.0	2.0	2.7	3.3	3.0	3.7	2.7	3.7	4.0	3.0	4.0	3.3	3.7	3.7	3.0	4.0	3.0	3.0	3.3	3.3
Edema	0	0	0	0	0	0	0	0	0	0	0	0	1.0	0.7	2.0	0	0	0	0	0	0	0	0	0	1.7	0	2.7	0	0	0	0	0	0	2.0	2.3	1.7
Injection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.0	1.7	2.0
Irritation score	3	2	2	2	3	2	1	2	2	3	2	2	9	6	9	5	6	3	4	6	5	7	7	8	10	7	11	7	7	7	7	8	7	12	11	11
Rating	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	++	+	++	+	+	+/–	+/–	+	+	+	+	+	++	+	++	+	+	+	+	+	+	+++	++	++
MIS	2			2			2			2			8			5			5			7			9			7			7			11		
Acceptability rating	Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Marginal			Acceptable			Acceptable			Unacceptable		

PE, proestrus; E, estrus; ME, metestrus; DE, diestrus; OVX, ovariectmized; MIS, mean irritation score. *Expected from the uterus, not typeable of estrus cycle of the vagina. Rating: +/–, minimal; +, mild; ++, moderate; +++, marked.

Table 2. Repeat-dose Vagina Irritation Study (4 Days)

Test article	Vaseline												SDS 5%												SDS 20%											
Group	PE			E			ME–DE			OVX			PE			E			ME–DE			OVX			PE			E			ME–DE			OVX		
Animal No.	131	132	133	141	142	143	151	152	153	161	162	163	171	172	173	181	182	183	191	192	193	201	202	203#	211	212	213	221	222	223	231	232	233	241	242	243
Estrus cycle of vagina	E	PE	PE	E	E	E	DE	ME	DE	–	–	–	DE	DE	DE	E	DE	DE	DE	ME	ME	–	–	E	E	DE	DE	ME	E	ME	DE	DE*	E	–	–	–
Findings																																				
Epithelium	0	0	0	0	0	0	0	0.7	0	1.0	1.0	1.0	1.0	1.0	2.7	0	2.0	1.0	1.0	1.0	0	1.0	2.0	0	4.0	3.7	3.0	4.0	2.7	3.0	3.0	4.0	4.0	4.0	4.0	3.0
Leukocytes	1.3	1.3	1.3	1.7	1.7	1.3	1.7	1.3	1.7	1.0	1.0	1.3	2.0	2.7	3.3	2.3	2.7	2.3	2.0	2.3	2.7	2.0	3.3	3.3	4.0	3.3	3.0	4.0	3.7	4.0	3.0	4.0	4.0	4.0	3.7	3.7
Edema	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.7	0	0	0.3	0.7	0	0	1.7	2.7	3.0	3.0	0.3
Injection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3	0.3	0.3	0.0	0.7	0	0.7	2.0	3.0	2.0	0.3
Irritation score	1	1	1	2	2	1	2	2	2	2	2	2	3	4	6	2	5	3	3	3	3	3	5	3	9	7	6	9	7	8	6	10	13	14	13	7
Rating	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+	+/–	+	+/–	+/–	+/–	+/–	+/–	+	+/–	++	+	+	++	+	+	+	++	+++	+++	+++	+
MIS	1			2			2			2			4			3			3			4			7			8			10			11		
Acceptability rating	Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Acceptable			Marginal			Unacceptable		

PE, proestrus; E, estrus; ME, metestrus; DE, diestrus; OVX, ovariectmized; MIS, mean irritation score. *Expected from the uterus, not typeable of estrus cycle of the vagina. Rating: +/–, minimal; +, mild; ++, moderate; +++, marked. #The score of this animal is for information only and is not included in the results because ovariectomy was judged to be incomplete in this animal.

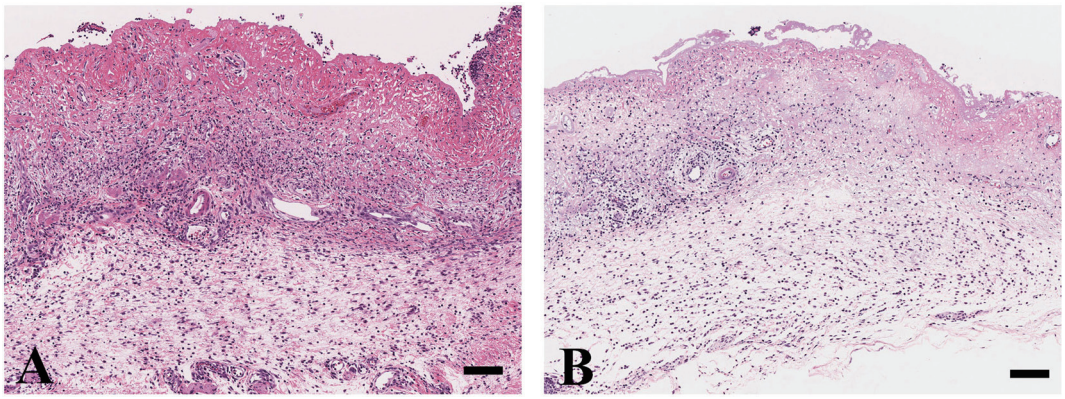

Fig. 3. Histopathological findings of the vagina in rats treated with 20% SDS in a repeat-dose vaginal irritation study. A and B: Severe inflammation, ulceration and necrosis of the vaginal mucosa, edema of the muscular and adventitial layers, and necrosis of the vessels in the lamina propria are observed (scores for A: epithelium, 4; leukocytes, 4; edema, 3; vascular injection, 3; ME-DE group) (scores for B: epithelium, 4; leukocytes, 4; edema, 3; vascular injection, 4; OVX group). H&E stain. Bars = 100 µm.

severe ulceration of the vaginal epithelium and inflammatory cell infiltration were observed; in addition, edema of the muscular and adventitial layers and necrosis of the vessels of the lamina propria were also noted (Fig. 3A and B). In other rats of each group treated with 30% SDS, minimal to severe ulceration of the vaginal epithelium, mild to severe inflammatory cell infiltration, minimal to moderate edema, and hemorrhage and/or necrosis of the vessels of the lamina propria were also observed, though gross findings were not observed. On the other hand, in most animals in each group treated with 5% SDS, degeneration of the vaginal epithelial cells and mild to moderate inflammatory cell infiltration were noted, but the degree of these findings was milder than that in each group treated with 5% SDS in the single-dose study.

The irritation score increased in an SDS dose-dependent manner in each group (PE, E, ME-DE, and OVX) (Table 2). At 20% SDS, varying irritation scores were observed in all the groups, and changes related to the estrus cycle were not observed in the PE, E, or ME-DE groups. The vaginal irritation scores of rats with gross findings were relatively high (scores: 10 = moderate and 13 = marked in the ME-DE group, 14 = marked in the OVX group). High vaginal irritation scores (9 = moderate) were observed in 1 of the 3 rats in each of the PE and E groups treated with 20% SDS, and a high irritation score (13 = marked) was observed in 1 of the 3 rats in the OVX group treated with 20% SDS, although gross findings were not observed in these animals. Lower irritation scores (6 to 8 = mild) were also observed in all groups treated with 20% SDS.

In the ME-DE group treated with 20% SDS, the MIS was higher than that in the PE or E groups treated with 20% SDS. However, this group had the highest (13) and lowest (6) irritation scores of all intact rats. The estrus cycle at the time of first administration was not correlated with the irritation score in any intact rats. In addition, variations in these irritation scores were also observed when the scores were added for all stages of the vaginal estrus cycle at the time of necropsy. The highest MIS was observed in the OVX group treated with 20% SDS; however, a lower irritation score (7) was also observed in this group.

In all intact rats (PE, E and ME-DE) treated with 5% SDS, the range of irritation scores was almost equivalent to that of the OVX rats, and the acceptability ratings of all intact rats and of OVX rats were both “acceptable” (Table 3 and 4). On the other hand, at 20% SDS, the range of irritation scores for all the intact rats was 6 to 13 (mild to marked), which was almost equivalent to that of the OVX rats (7 to 14, mild to marked) (Table 3), although the acceptability rating varied according to group (intact rats, acceptable; OVX, unacceptable) (Table 4).

Discussion

In this study, we evaluated the irritancy of the vagina in intact rats in relation to each stage of the estrus cycle and compared the results with the irritancy of the vagina in OVX rats, which exhibit a late diestrus-like condition. We used 5% SDS and 20% or 30% SDS as the irritant. The higher concentration of SDS caused severe irritancy, including the exfoliation of the vaginal epithelium, inflammatory cell infiltration, and edema. After the 5% SDS treatment, the irritation ratings of all the intact and OVX rats were minimal to mild. SDS (also known as sodium lauryl sulfate, SLS) is an alkyl sulfate that is commonly used in research applications and in commercially available personal hygiene products, and it’s irritancy and resulting inflammatory response have been reported using patch tests in humans[9, 10]. The concentration of SDS in this study was considered to be appropriate for evaluating vaginal irritancy, since the irritant reactions induced by a 10% aqueous SLS solution and the inflammatory response in the epidermis induced by a 5% aqueous SLS solution have been previously reported[9, 10].

OVX rats are considered to be a sensitive model of both single- and repeat-dose irritation studies. It has been reported that OVX rats exhibit a late diestrus-like condi-

Table 3. Irritation Score and Rating of Intact and OVX Rats (Repeat-dose Vaginal Irritation Study)

	Vaseline	SDS 5%	SDS 20%
Intact rats (n = 9)	1–2 (minimal)	2–6 (minimal-mild)	6–13 (mild-marked)
OVX rats (n = 3)	2 (minimal)	3–5 (minimal-mild)*	7–14 (mild-marked)

*n = 2.

Table 4. Mean Irritation Score and Acceptability Rating of Intact and OVX Rats (Repeat-dose Vaginal Irritation Study)

	Vaseline	SDS 5%	SDS 20%
Intact rats (n = 9)	2 (acceptable)	4 (acceptable)	8 (acceptable)
OVX rats (n = 3)	2 (acceptable)	4 (acceptable)*	11 (unacceptable)

*n = 2.

tion; however, we demonstrated that the vaginal epithelium of OVX rats was relatively thin, consisting of two or three cell layers, and many degenerated epithelial cells were visible. Ultrastructurally, the vaginal epithelial organization of ovariectomized rats is known to be significantly degenerated with severely dilated intercellular junctions[11]. These observations suggest that the irritancy of the vagina in OVX rats might be related to relative ease of permeation of the test article and that the vaginal epithelium might be overly sensitive to irritation.

Vaginal irritancy in the intact rats varied according to the stage of the estrus cycle at the time of administration in the single-dose vaginal irritation study. The irritation score of the PE group treated with SDS was higher than that of the E or ME-DE group. In the PE group, exfoliation of the epithelium, severe inflammatory cell infiltration, and edema of the muscular and adventitial layers in the vagina were histopathologically noted, and the irritation score of the PE group was consistent with the histopathological findings. In the E and ME-DE groups, focal ulceration and inflammatory cell infiltration were noted, but the grades and irritation scores were lower than those in the PE group. The vaginal epithelium varies widely during the normal estrus cycle. The vaginal epithelium is composed of a superficial mucoid layer during proestrus, and these surface cells are covered with microvilli ultrastructurally[12, 13]. On the other hand, the surface layer is cornified in the estrus stage, and in the metestrus to diestrus stages, the vaginal epithelium is approximately 3–7 cells thick[6]. During these stages, the surface cells are smooth ultrastructurally or have a flat membrane with no microvilli appearance[12, 13]. When considering the vaginal morphological features of these stages, the present results indicating that the irritation score of the PE group treated with SDS was higher than that of the E or ME-DE group make a great deal of sense. These morphological differences suggest that the irritancy might be related to the presence of microvilli and the wide surface area because of the extended stagnation of the test article in the vagina. High irritation scores were observed in 2 of the 3 animals in the PE group treated with 5% SDS, while the irritation scores in the OVX group treated with 5% SDS were mild for all the animals. On the other hand, the irritation scores of the PE group were lower than those of the OVX group when treated with 30% SDS. The acceptability rating of the PE group was "marginal," while that of the OVX group was "unacceptable." These results suggest that evaluations of vaginal irritancy in a single-dose study require identification of the estrus cycle at the time of administration. In addition, evaluations using intact rats should be performed taking into consideration the vaginal irritancy of the proestrus stage, which might exceed that of OVX rats.

In the 4-day repeat-dose vaginal irritation study, the vaginal irritancy of the intact rats showed specific features in each group, but no remarkable differences in the irritation scores were observed among the PE, E, and ME-DE groups. In the ME-DE group treated with 20% SDS, the MIS was higher than that in the PE or E groups treated with 20% SDS. However, the irritation scores of the individual animals in this group included the highest and lowest scores among all intact rats. In addition, edema, which caused the irritation score to be high, was observed in the PE, E, and ME-DE groups (1 of the 3, 2 of the 3, and 2 of the 3 rats, respectively); therefore, the higher MIS in the ME-DE group was considered to be affected by large variation of individual irritation scores and especially by the individual showing the highest score. Furthermore, the vaginal estrus cycle at the time of necropsy was histologically determined to be at the estrus stage in 3 rats, and edema was observed in 3 of the 3 rats at this stage. The animals at the estrus stage at the time of necropsy were considered to be at the proestrus stage at the last administration on the day before necropsy. Therefore, edema might be related to the stage of the estrus cycle at the proximate administration, since the proestrus stage was estimated to be more sensitive to irritation from the results of the single-dose study. Among the 3 rats showing edema in the estrus stage at the time of necropsy, the individual irritation scores were mild to marked (irritation scores: 7, 9, and 13), and there was also large variation. These results of the repeat-dose vaginal irritation study suggests that the stage of the estrus cycle at the time of necropsy was

not correlated with the stage of the estrus cycle at the time of administration four days earlier and that the stage of the estrus cycle at the time of necropsy was also not correlated with the irritation score. Proestrus reportedly lasts approximately 12 hours, while estrus lasts 9 to 15 hours, metestrus lasts 21 hours, and diestrus lasts 57 hours[14]. Based on this information, repeat administration can be considered to result in an "averaged" irritancy value, and it is suggested that evaluation of vaginal irritation without confirmation of the stage of the estrus cycle is possible in a repeat-dose study.

Comparing the irritation scores in all intact rats and OVX rats, irritation was evaluated as minimal for Vaseline, minimal to mild for 5% SDS, and mild to marked for 20% SDS in both intact and OVX rats (Table 3), and the range of irritation scores was similar between intact rats and OVX rats in each treatment group. These results suggests that intact rats might be useful for evaluating vaginal irritancy, without requiring complicated protocols or techniques for ovariectomy causing the rats additional pain. Though the acceptability rating calculated from each irritation score for all intact rats treated with 20% SDS was "acceptable," individual evaluation showed mild to marked vaginal irritancy. Therefore, individual irritation scores should be evaluated in addition to the MIS when performing vaginal irritation studies, and they are expected to enable reliable evaluations of a compound's irritation potential.

Comparing the irritancy of each group treated with 5% SDS in the single-dose study to that in the repeat-dose study, the irritation scores in the single-dose study were higher than those in the repeat-dose study. The skin is known to exhibit an accommodation phenomenon following repeated exposure to an irritant[15]. In the repeated vaginal irritation study with continuous exposure, a similar phenomenon may have occurred in the vagina, though the accommodation phenomenon has not previously been reported for vaginal irritancy.

In summary, the present study suggests that identification of the estrus cycle is needed for single-dose vaginal irritation studies, as irritancy was affected by the stage of the estrus cycle on the day of administration in rats. On the other hand, it also suggests that evaluation without confirmation of the estrus stage is acceptable in a 4-day repeat-dose vaginal irritation study, as the range of irritation scores in the intact rats, with irritancy being averaged by repeat administration throughout the estrus cycle, was similar to that of the OVX rats. Thus, the present study demonstrated that intact rats are well suited as a model for evaluating vaginal irritancy and as an alternative to OVX rats.

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