2.2％ブドウ糖)またはHRG(1/2リンゲル液＋2.5％ブドウ糖)群に分け，一日水分維持量である30 ml/kgの各輸液剤を20 ml/kg/時で静脈内投与した．ILGの静脈内投与によりCVPは著しく上昇し，投与中は6.0から9.0 mmHgの高値を維持した．一方，HLGおよびHRG群のCVPは輸液剤投与による有意な変動は認められなかった．投与前値に対する予備塩基濃度の変化量(rBE)はILGおよびHRGいずれの静脈内投与によっても有意に減少し，HRG群では投与終了後も低値を維持した．一方，HLG群におけるrBEの減少は軽度で，投与終了後には投与前値よりも高値となった．従って，一日水分維持量の低張輸液剤を20 ml/kg/時の投与速度で急速静脈内投与しても，安全性に問題はないと思われた．また，HLGは輸液療法に伴う希釈性アシドーシスを予防するだけでなく，輸液後に軽度の代謝性アシドーシスを是正するため，HLGの維持量急速静脈内投与は脱水および軽度の代謝性アシドーシス牛の輸液療法において有用であると思われた．

臨床繁殖学：

1998年8月から1999年7月にかけて，3頭の非妊娠，雌マレーグマ(*Helarctos malayanus*)を対象に糞中から検出されるエストラジオールの定量を，マレイシア国サラワク州において実施した．また，それに加えて膣粘液中に見出される細胞の観察を1998年8月と1999年3月にマレーグマ1頭を対象に行った．3頭の糞中エストラジオール濃度は，1998年8月または9月にピークが観察された．また，膣粘液中に観察される角化上皮細胞の出現割合は，1998年8月の数値が1999年3月のものより高かった．これらのことからマレイシア国サラワク州において，マレーグマはエストラジオールの濃度がピークをむかえる8月から9月ごろに発情している可能性が示唆された．

ヒグマの射出精液を最終濃度4.7％のグリセリンを添加したトリス－卵黄－クエン酸緩衝液を用いて凍結した．80分間のグリセリン平衡処理後0.25 mlストローに充填した精液は液体窒素蒸気で冷却したのち，6～58日間液体窒素中に保存した．融解精子の運動率，生存率および活発な直進運動を示す割合は，平均43，67および19％であった．また，活発な直進運動を示す精子数は射出精液当たり1.8×10^8個であった．

HatanoラットはSprague-Dawley系ラットからシャトルボックス回避学習試験の結果を指標に分離された2系統の近交系(高回避および低回避ラット)であり，精子運動性に系統差が認められている．今回，精子の形態観察を行ったところ，運動率の低い低回避ラット精子は精子奇形率が高回避ラット精子より3倍以上高かった．低回避ラットに認められた精子奇形率の高さはラットでは珍しく，精子性状に関するモデル動物となる可能性が示唆された．

毒　性　学：

幼若雌Crj:CD®(SD)IGSラット(21-36日齢)について一般状態観察，体重を測定し，膣開口日齢を検査した．動物は各日齢で解剖し，解剖日に子宮，卵巣重量測定及び血清

中の 17β-estradiol (E2), progesterone 測定を行った．28 日齢以降みられた E2 濃度の上昇に始まり，体重を除く全ての検査項目が 31 日齢までに明らかな変化を示した．腟開口は 34 日齢に最も高頻度にみられた．剖検所見においては，腟開口がみられていないラットの多くで子宮の imbibition が観察された．解剖時に腟開口が観察されたラットの子宮，卵巣を病理組織学的に検査したところ，卵巣にはすでに黄体形成が確認され，子宮の所見は発情後期の像を呈していた．以上の結果，雌 Crj:CD®(SD)IGS ラットの内分泌撹乱化学物質 *in vivo* スクリーニング試験のための生殖器の発達に関する貴重なデータが得られ，また初回排卵は，腟開口の直前又は腟開口と同時に起こると推察された．

FULL PAPER *Toxicology*

Age-Related Changes of Genital Systems in the Female Crj:CD® (SD) IGS Rats during Sexual Maturation

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ABSTRACT. The age-related changes of vaginal opening, body weight, the weights of the uterus and ovary, together with histological examination, serum 17β-estradiol (E2) and progesterone levels were examined in intact female Crj:CD® (SD) IGS rats between 21 and 36 days of age to understand the basic biological profile of changes of the female genital system during sexual maturation in the rat for female pubertal assays. With the beginning of the elevation of serum E2 level from 28 days of age, all parameters except body weight started to show drastic change until 31 days of age. The highest incidence of vaginal opening was recorded at 34 days of age. On macroscopic examinations, a number of rats showed uterine imbibition but vaginal opening. Immediately after the confirmation of the vaginal opening, the genital systems of three rats were observed microscopically. Both ovaries already had multiple corpora lutea, and degeneration of endometrial epithelial cells was observed. In conclusion, we obtained essential data on genital tract development of female Crj:CD® (SD) IGS rats for *in vivo* screening assays that will contribute to detect potential endocrine active chemicals. In addition, it is assumed that the first ovulation precedes or occurs simultaneously with vaginal opening.

KEY WORDS: Crj:CD® (SD) IGS female rat, female pubertal assay, first ovulation, pubertal onset, vaginal opening.

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Currently, there is much concern that certain environmental chemicals may have the potential to disturb normal sexual differentiation and development in wild life and humans [3, 8, 9]. A uterotrophic assay using immature female rats was proposed by the Organization for Economic Cooperation and Development and Endocrine Disrupter Screening and Testing Committee (EDSTAC) of the U.S. Environmental Protection Agency as an *in vivo* screening method to detect the estrogenic or anti-estrogenic activity of the chemicals acting mainly by receptor-mediated mechanisms [6, 7]. EDSTAC has recommended another assay, termed the "female pubertal assay" [5]. The purpose of this assay is to quantify the effects of environmental chemicals on pubertal development and thyroid function in the immature female rat. Focusing on disruption of the sex hormone system, the proposed endpoints of this assay are the age of vaginal opening, reproductive organ weights and sex-related hormone levels. Which strain is best for these assays is currently uncertain, because there are differences in sensitivity among rat strains [12, 13]. Crj:CD® (SD) IGS rat (SD IGS rat) was developed recently under a new breeding system for the purpose of supplying experimental animals with minimal genetic variations. Many researchers have begun to use for regulatory toxicology studies and for various types of biochemical research. However some general biological parameters of this rat have been reported, development of the female genital system in SD IGS rats during peripubertal period has not been well characterized. An understanding of the basic biological profile of changes in the female genital system during sexual maturation in the rat is of importance for analyzing the results of female pubertal assays. In the present study, we determined age-related changes in vaginal opening, body weight, the weights of the uterus and ovary together with gross pathological examination, serum 17β-estradiol (E2) and progesterone levels of intact non-treated SD IGS female rats from 21 to 36 days of age, i.e., during the pubertal period. In addition, the genital systems of three rats immediately after vaginal opening were examined microscopically to obtain the preliminary data on the relationship between the first ovulation and vaginal opening.

MATERIALS AND METHODS

Animals: Fourteen timed-pregnant specific-pathogen-free female SD IGS rats were purchased from Charles River Japan, Inc. (Hino Breeding Center and Atsugi Breeding Center). Insemination was confirmed by the presence of a sperm-plug in the vagina. The day following overnight mating was designated as pregnant day 0 and the rats were primiparous at 12 weeks of age. They arrived on pregnant day 14. The animals were housed in an animal room with the temperature set at 23 ± 2°C, the relative humidity at 55 ± 10%, the ventilation rate at 10–15 times/hr, and lighting for 12 hr daily from 7:00 am to 7:00 pm. Dams were housed individually in hanging stainless steel cages with a wire-mesh floor (260 W × 380 D × 180 H mm) from pregnant day 14 to 17. Neonatal rats were delivered in our laboratory (date of birth designated 0 days of age). Dams and their litters were housed in polycabonate cages (280 W x 440 D x 150 H mm) with nesting materials (Sun Flake®, Chiba Animal Material Co., Ltd., Japan) from pregnant day 17 until weaning. At 4 days of age each litter was culled to eight female rats. The rats were weaned at 20 days of age. Then, weanlings were ranked by weight and 20 rats each were randomly assigned into 12 experimental groups using a body-

weight stratified randomization method to minimize variation in body weights among the groups. After grouping, the weanlings were housed in stainless steel cages with a wire-mesh floor (165 W × 300 D × 150 H mm) hung in a 6-vertical by 6-horizontal cage allocable stainless steel cage rack (1 animal/cage). The animals were allowed free access to autoclaved solid food (before weaning of offspring, CRF-1, after weaning, MF, Oriental Yeast Co., Ltd., Tokyo, Japan), and to chlorinated tap water from an automatic dispenser or supply bottles. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *the Japanese Association for Laboratory Animal Science.*

Experimental designs: The twenty intact rats in each group were sacrificed at 21, 23, 24, 28, 29, 30, 31, 32, 33, 34, 35 or 36 days of age. At necropsy, the rats were weighed and blood samples were collected from the abdominal aorta and then euthanized by exanguination under ether deep anesthesia. To reduce the fluctuation of hormonal levels due to stress, the transportation and handling of rats were carried out with great care on the day of necropsy. Macroscopic examinations of the female rat genital system were performed. As 3 rats showed vaginal opening immediately before dissection and corpora lutea-like structures were observed macroscopically, the genital systems of these rats were examined microscopically; the samples were prepared by fixation in neutral-buffered formalin after embedding in paraffin, followed by sectioning and hematoxylin and eosin staining to confirm that they had corpora lutea. The ovaries and uterus of each rat were removed and weighed. The blotted uterine weights, i.e., weight without excess fluid, were also determined by excising the uterine horns and blotting the excess fluid onto filter paper. The sera were stored at –80°C until measurement of 17β-estradiol (E2) and progesterone levels. Until sacrifice, general condition and evidence of vaginal opening were recorded daily and body weight was determined once a week from 21 days of age. The serum E2 level was measured using a commercial radioimmunoassay kit (DPC estradiol double antibody kit, lot No. 300, Diagnostic Products Co., Los Angels, CA, U.S.A.), and serum progesterone was measured using a commercial immunoassay kit (Progesterone Enzyme Immunoassay Kit, lot No. 13153A, ©Cayman Chemical Company, Ann Arbor, MI, U.S.A.).

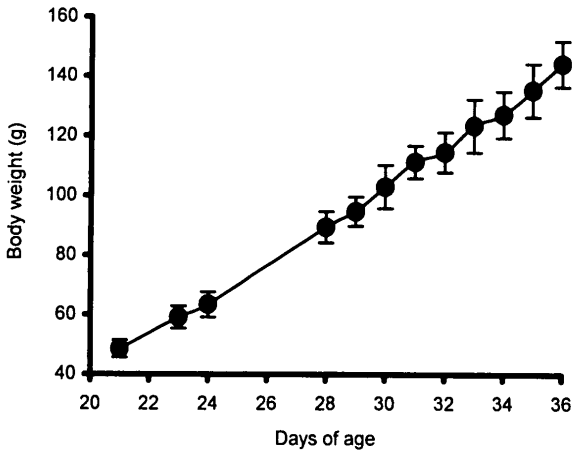

Fig. 1. Body weights of female SD IGS rats during the pubertal period. Points are means and vertical lines represent SD.

RESULTS

General conditions and body weights: During daily clinical observations, no abnormalities were noted and mean body weight gradually increased (Fig. 1). The mean value ± standard deviation (SD) of the body weight at weanling 20

Table 1. Vaginal opening in SD IGS rats during pubertal period

	Days of age					
	30	31	32	33	34	35
Number of vaginal opening/examined	1/140	8/120	12/100	3/80	23/60	10/40
Percentage (%)	0.7	6.7	12.0	3.8	38.3	25.0

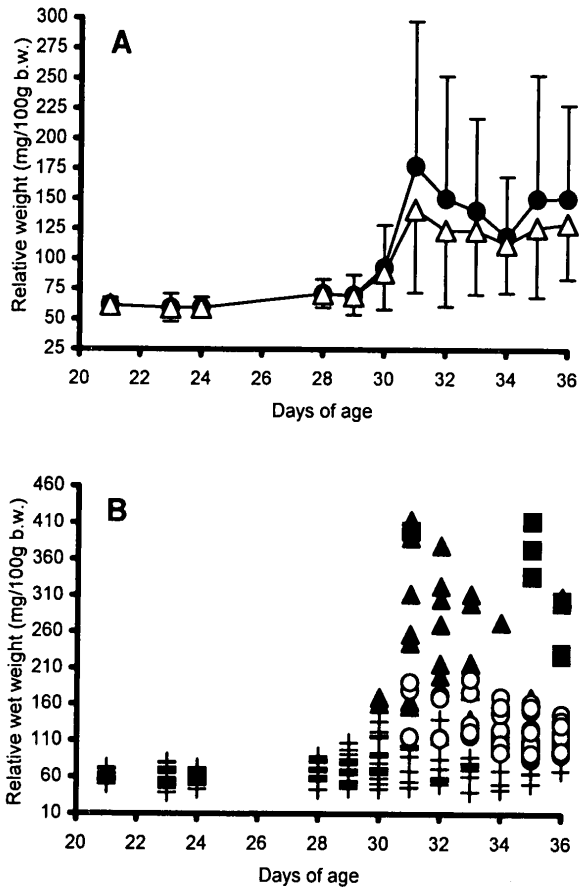

Fig. 2. Relative uterine weight in female SD IGS rats during pubertal period. (A), Uterine weight. Points are means and vertical lines represent SD (n=20). ●, wet weight; △, blotted weight. (B), Individual uterine weights with genital system findings. +, rat with neither vaginal opening nor uterine imbibition; ▲, without vaginal opening but with uterine imbibition; ○, with vaginal opening but without uterine imbibition; ■, with both vaginal opening and uterine imbibition.

days of age was 48.5 grams ± 2.9.

Vaginal opening: The earliest incidence of vaginal opening occurred at 30 days of age, the latest at 35 and the highest at 34 (Table 1).

Organ weights and findings: In general, weight of uterus increased slightly until 29 days of age and then increased rapidly, first peaking at 31 days of age, and was associated with the weight fluctuation (Fig. 2A). The profiles of the absolute uterine weights were similar to relative weights (Data not shown). On macroscopic examinations, watery contents in the uterine lumen, i.e., uterine imbibition, was observed in rats older than 30 day. These findings were considered to be identical to those of the proestrus stage. Many rats showed uterine imbibition but not vaginal opening (Fig. 2B, closed triangles.). On the other hand, some rats with uterine imbibition already showed vaginal opening (Fig. 2B, closed squares) occurring at later ages during our experimental period, i.e., 35 or 36 days of age, except in one animal. Absolute ovarian weights gradually increased until 28 days of age and thereafter more increased (Fig. 3A). On the other hand, relative weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age followed by an increase from 30 to 34 days of age (Fig. 3B).

Three rats were dissected immediately after vaginal opening was achieved. Figure 4 presents an example of the microscopic findings of genital system when immediately vaginal opening was observed, but it could not found at the routine morning observation. The interval between the time of morning observation and the discovery of vaginal opening was approximately 3 hr. Both ovaries had multiple corpora lutea consisting of cells with scant cytoplasm containing a small number of fine vacuoles, indicating the corpora lutea to not yet be mature (Fig. 4A). A large propor-

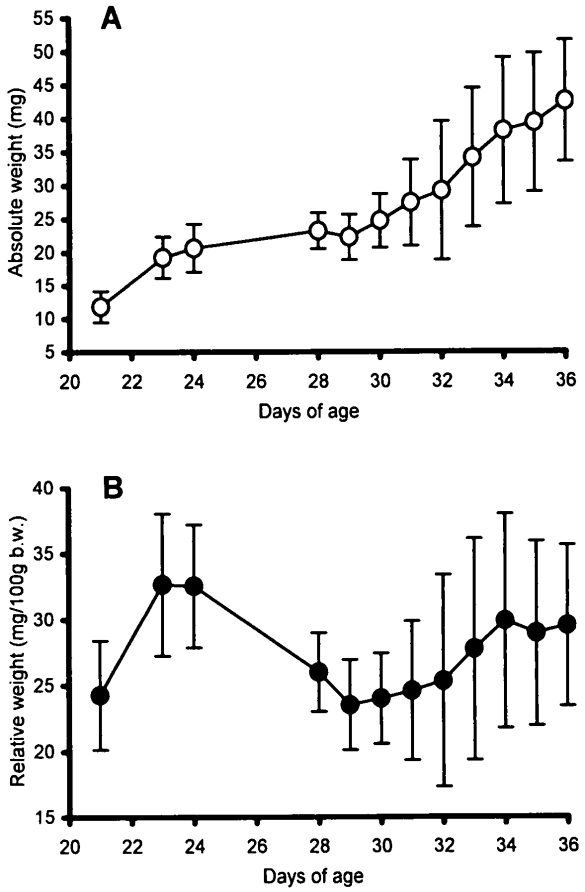

Fig. 3. Ovarian weight in female SD IGS rats during pubertal period. (A), Absolute ovarian weight. (B), Relative ovarian weight. Points are means and vertical lines represent SD (n=20).

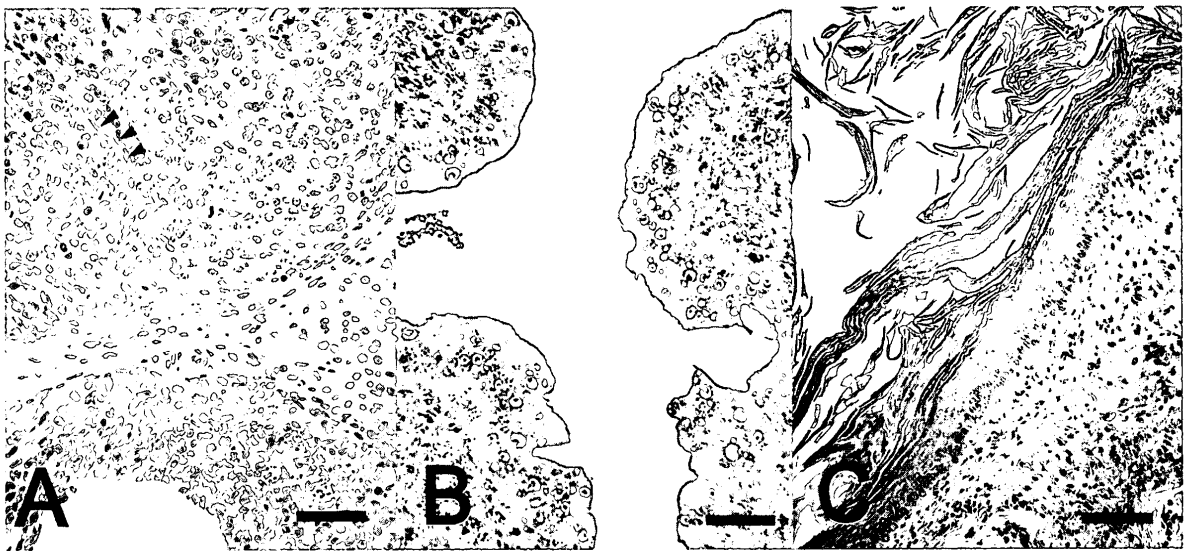

Fig. 4. Microscopic findings of the genital systems of the rat immediately after vaginal opening was noted. (A), Ovary showing a corpus luteum (upper area) and a Graafian follicle (lower area). Arrowheads point a blood vessel, which is a feature of corpora lutea. Cells with relatively rich cytoplasms and prominent nucleoli tightly contact with each other. In contrast, a Graafian follicle consists of granulosa cells with dense nuclei and occasional loose intercellular gaps. H&E staining. Bar=50 μm. (B), degeneration of endometrial epithelial cells. H&E staining. Bar=50 μm. (C), Prominent cornification in vagina. H&E staining. Bar=100 μm.

tion of endometrial cell was degenerative, as in the metestrus phase of the endmetrium of normal mature rats (Fig. 4B). In the vagina, cornification, matching the late estrus phase was evident (Fig. 4C).

Serum E2 and progesterone levels: The high initial E2 level at 21 days of age decreased at 28 days of age. Generally, the first peak was seen at 31 days of age, followed by a gradual decrease until 34 days of age (Fig. 5A). The serum E2 level variation after 29 days of age was very large, as was that of uterine weight. There was a significant positive relationship between the serum E2 level and uterine weight after 29 days of age (r=0.724, p<0.001). Serum E2 levels tended to be higher in rats with uterine imbibition than in those without it (Fig. 5B). After 31 days of age, serum progesterone levels increased rapidly (Fig. 6).

DISCUSSION

In this study, we measured changes in the frequency of vaginal opening, uterine weight, ovarian weight, and both serum E2 and progesterone levels, seen during the pubertal period in female SD IGS rats.

With the beginning of the elevation of serum E2 level from 28 days of age, uterine and ovarian weights, serum progesterone level started to show drastic change until 31 days of age. Vaginal opening and uterus imbibition was also observed in rats older than 30 days. Especially, uterine weight change correlated with the serum E2 level after 29 days of age. E2 is known to increase uterine weight and promote hypertrophy of the endometrial epithelium [2]. These changes after 28 days of age were considered to originate in pubertal onset. In general, pubertal onset in rats encompasses the period of vaginal opening and first ovulation [4]. In female SD IGS rats of this study, pubertal onset was considered to start from 28 days of age. It is noteworthy that many rats showed uterine imbibition without vaginal opening. This means that uterine imbibition, i.e., proestrus-like change, precedes vaginal opening.

The high initial E2 level at 21 days of age did not have an effect on the uterine weight. This is attributable to the high concentration of α-fetoprotein, which binds up the estrogens available around this age [1, 10]. It is generally accepted that E2 is inactive when bound to α-fetoprotein. In addition, relative ovarian weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age. Meijs-Roelofs *et al.* reported that the number of follicles with a volume $\geq 100 \times 10^5\ \mu m^3$ increased rapidly until 23 days of age and then more slowly until 27 days of age in contrast to the gradual body weight increase [11]. Thus, the decrease in relative ovarian weight after 24 days of age may be attributable to altered numbers of follicles at this stage.

In addition to the experiment described above, three rats were dissected immediately after vaginal opening was achieved. The histological findings of the genital systems of these rats, especially in terms of the corpora lutea formation and degenerative endometrium are indicative of the metestrus stage. These findings lead us to speculate that the

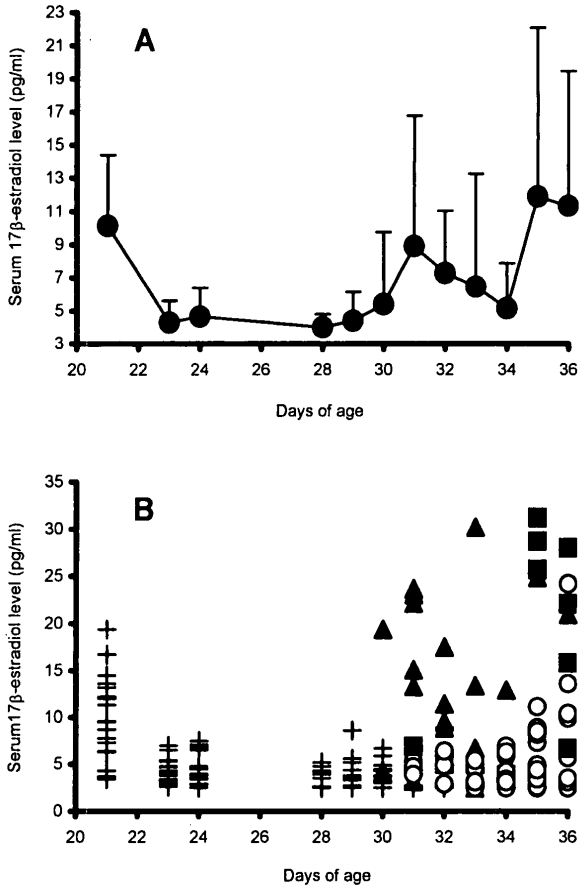

Fig. 5. Serum 17β-estradiol (E2) levels in female SD IGS rats during pubertal period. (A), Serum E2 level. Points are means and vertical lines represent SD (n=20). Detection limit, <2.5 *pg/ml*, was excluded from the calculation. (B), Relationship between individual serum E2 levels and gross findings. +, rat with neither vaginal opening nor uterine imbibition; ▲, without vaginal opening but with uterine imbibition; ○, with vaginal opening but without uterine imbibition; ■, with both vaginal opening and uterine imbibition.

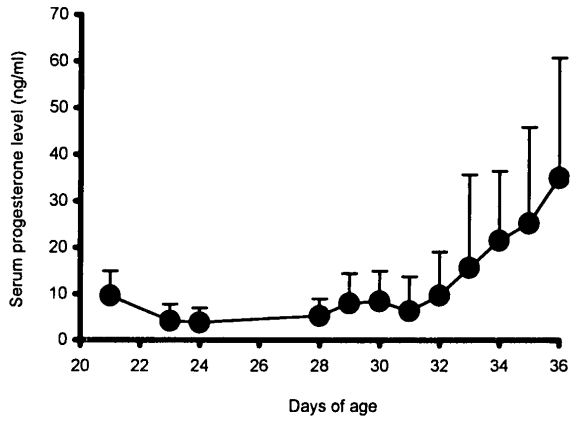

Fig. 6. Serum progesterone level in female SD IGS rats during pubertal period. Points are means and vertical lines represent SD (n=20).

first ovulation in the rat could occur before or at least at the same time as vaginal opening because it is unlikely that multiple corpora lutea formation and endometrial degeneration could occur in such a short interval. This hypothesis is supported by our observation that proestrus-like change precedes vaginal opening. It is noteworthy that our hypothesis contrasts with the literature, in which, vaginal opening is generally described as occurring with or shortly before the first ovulation [4]. However, we have not pinpointed the histology of the genital systems of these rats at the instant of vaginal opening nor have we clarified whether the histological findings of the rat genital system at the instant of vaginal opening are identical to those of mature and normally cycling rats. The further studies will be needed.

In conclusion, we obtained essential data on genital tract development of female SD IGS rats for *in vivo* screening assays that will contribute to detect potential endocrine active chemicals. Our results will contribute to analyses of studies employing the peripubertal female rats. In addition, it is assumed that the first ovulation precedes or occurs simultaneously with vaginal opening, which opposes the established theory in terms of the timing of the ovulation.

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