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Article

Pueraria *mirifica* Exerts Estrogenic Effect and Promotes Mammary and Endometrial

Running title: Effect of Pueraria *mirifica* on Mammary and Uterine Carcinogenesis in Rats

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Abstract: Pueraria *mirifica* (PM) is a plant which dried and powdered tuberous root is now widely used as a rejuvenating herb to promote youthfulness in both men and women. In this study, we investigated the modifying effects of PM at various doses on mammary and endometrial carcinogenesis in female Donryu rats. Firstly, PM administered to ovariectomized Donryu rats at doses of 0.03, 0.3 and 3% in phytoestrogen-low diet for 2 weeks induced the significant increases of uterus weight. Secondly, 4-week PM application to non-operated rats at a dose of 3% after the 7,12-dimethylbenz[a]anthracene (DMBA) initiation, resulted in significant elevation of cell proliferation in the mammary glands. In the third experiment, postpubertal administration of 0.3% (200 mg/kg b.w./day) PM to 5-week-old non-operated animals for 36 weeks following the initiation of mammary and endometrial carcinogenesis with DMBA and N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG), respectively, caused significant increases of mammary adenocarcinoma incidence and multiplicity. A trend for increase of uterine adenocarcinomas and a significant increase of endometrial atypical hyperplasia multiplicity was observed at 0.3% PM. Furthermore, PM at doses of 0.3 and mostly 1% induced dilatation, hemorrhage and inflammation of the uterine wall. In conclusion, the postpubertal long-term PM administration to Donryu rats exerted estrogenic effect in the mammary gland and uterus, and at a dose of 200 mg/kg b.w./day promoted carcinogenesis initiated by DMBA and ENNG.

Keywords: Pueraria *mirifica*; mammary gland; uterus; carcinogenesis; estrogenic activity; Donryu rat

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Introduction

Pueraria *mirifica* (PM), also known as white Kwao Krua, is a plant found in northern and north eastern Thailand which belongs to the family of Leguminosae, subfamily Papilionoideae or the soy, bean and pea subfamily. Previously, the evidence of PM application for the treatment of a range of conditions such as those related to the aging process has been reported [1]. The dried and powdered tuberous root of PM contains at least 17 chemical compounds with estrogenic biological activities, which are usually divided into 3 groups: The first group includes teniso flavonoids such as genistin, genistein, daidzein, daidzin, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puemiricarpene [2,3]. The second group contains coumestrans, comprised of coumestrol, mirificoumestan, mirificoumestanglycol and mirificoumestan hydrate. The third group included chromenes, such as miroestrol, deoxymiroestrol and isomiroestrol [2]. All these substances are the phytoestrogens with structures similar to that of 17β-estradiol. However, there are still contradictory reports for the presence of miroestrol, a phytoestrogen with the highest estrogenic activity among all the subsequentially isolated phytoestrogens from PM, which was considered similar to the estriol, known as safest estrogen for humans [4-7]. Furthermore, PM was reported to contain phytoestrogens β-sitosterol, stigmasterol, campesterol, and the cytotoxic non-phytoestrogen spinasterol [3,8]. The puerarin has been previously accounted for about half of the total isoflavone content of PM with a slightly comparable amount of genistin and daidzin. Genistin, daidzin and puerarin are isoflavones in glycoside forms and could be partially hydrolyzed in the intestine by cleaving a C-glycosyl bond to the aglycoside forms of genistein, daidzein, and daidzein, respectively [2].

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Nowadays, PM is available in tablets, extracts, cream, sprays and powdered form, can be added to some other medicinal preparations with other herbs, and each ailment requires different type of application and dosage [1]. It could be easily obtained from internet resources in many countries including USA and Japan, and is usually used for an effective anti-aging, supporting memory, smoothing the skin, preventing the parasite diseases, increasing hair growth, enlarging breast, improving appetite, and providing relief for ailments like osteoporosis and even cancer [9-12]. Continuous administration of PM at 20-100 mg/day for six months, or at 100-200 mg/day for 12 months, helped women having menopause symptoms, while no significant changes were detected in their hepatic, hematologic and renal functions [3]. Pretreatment with PM at high dose (1000 mg/kg body weight (b.w.)/day) for 4 weeks was shown to suppress the development of mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) in Spargue-Dawley rats [13]. However, the effect of long-term administration at different doses has not been yet investigated. Based on previous studies, a safe dosage of PM as a dietary supplement for humans was suggested at 1-2 mg/kg b.w./day or about 50-100 mg/day [3]. Nowadays, doses of 20 to 100 mg/day are commonly used, but in some cases 200-900 mg/day or even higher (up to 3000 mg/day) are applied. Till now no serious side effects have been recorded with the prescribed safe dosage, however, at high doses PM may cause epilepsy, diabetes, asthma and migraine [3].

Even there are a lot of data on the benefits of PM still there is a doubt that the herb, which exhibits strong estrogen-like properties, may stimulate the growth of existing estrogen-sensitive breast or endometrial tumors, pointing into question which dose is really safe. Previously, the nanomolar concentrations of genistein, which is one

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of PM ingredients, was shown to induce acid ceramidase (ASAH1) transcription through a GPR30-dependent, pertussis toxin-sensitive pathway that requires the activation of c-Src and extracellular signal regulated kinase 1/2 (ERK1/2), thus stimulating breast cancer cell growth [14]. Recently, we demonstrated that postpubertal administration of soy isoflavones in estrogenic dose promotes mammary and endometrial carcinogenesis in Donryu rats [15]. These data call into question the safety of a long-term administration of phytoestrogens with regard to the mammary gland and endometrium. It is of particular importance to detect the concentrations of PM which might exert promoting effects on mammary gland and uterine carcinogenesis. Therefore, the present study was carried out to investigate the modifying effects of various doses of PM on mammary and uterine endometrial carcinogenesis using the Donryu rat model.

Results

Estrogenic effect of test compounds (short-term exp. 1). After ovariectomy, PM treatment at doses of 0.3%, 3% and IA at a dose of 0.2%, resulted in cornification evident on examination of vaginal smears (Fig. 1).

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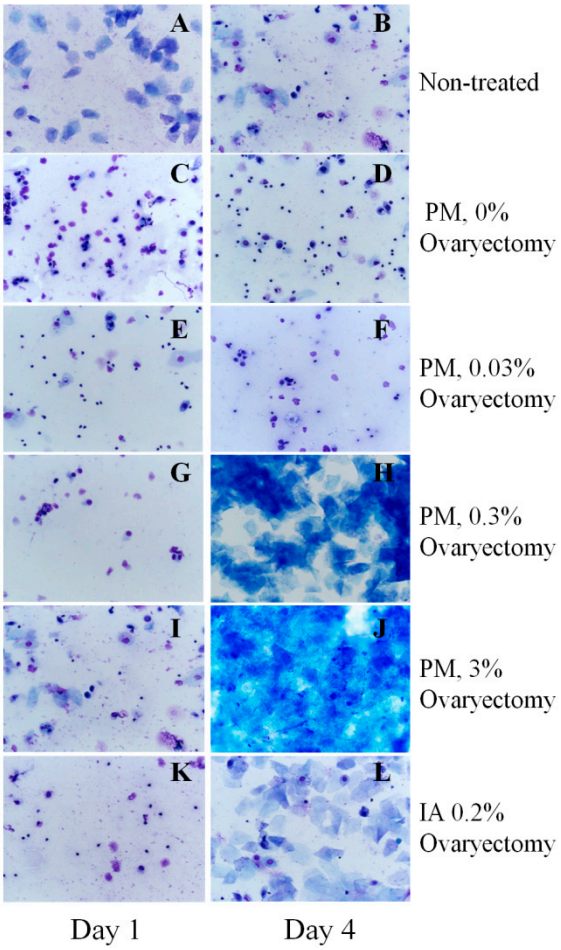

Fig. 1. Vaginal cytology in the non-treated (A,B) and ovariectomized (C-L) female Donryu rats administered PM and IA for the first 4 days. Animals were given PM at doses of 0 (C, D), 0.03 (E, F), 0.3 (G, H) and 3% (I, J), or the 0.2% IA (K, L) 2 weeks after the ovariectomy. Vaginal smears were obtained daily before and after starting the treatment, dried and stained with aqueous solution of methylene blue. In the ovariectomized rats the absence of cyclicity was confirmed by castration smear similar to status of diestrus. Note, that 0.3 and 3% PM, as well as 0.2% IA rats exerted estrogenic activities confirmed by cornification which was similar to estrus status.

The 0.3% PM treatment was found to exert even stronger estrogenic activity than the 0.2% IA. Mean relative uterus weight was significantly elevated in 0.03% (0.13±0.01%, $P<0.05$), 0.3% (0.31±0.03%, $P<0.05$), 3% PM (0.35±0.08%, $P<0.05$) and 0.2% IA (0.19±0.04%, $P<0.05$)-administered ovariectomized rats as compared to the control (0.08±0.01%). Thus, weak, medium and strong estrogenic activities of PM at doses of 0.03% (20 mg/kg b.w./day), 0.3% (200 mg/kg b.w./day) and 3% (2,000 mg/kg

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b.w./day), respectively, were demonstrated in the rat uterus. In this experiment, the significant decreases of body weights were observed in the 0.3% and 3% PM groups ($P<0.001$) as well as 0.2% IA group ($P<0.01$). Because of the too strong estrogenic effect of 3% PM detected in the short-term study, in the next long-term experiment the dose was changed from 3 to 1%. Thus, the test doses of PM in experiment 3 were set as low: 0.03%, medium: 0.3% and high: 1%.

Cell proliferation in the mammary gland (short-term exp. 2). BrdU immunohistochemistry revealed a dose-dependent increase of cell proliferation in the terminal end buds of the mammary gland of PM-treated rats after 4 weeks of

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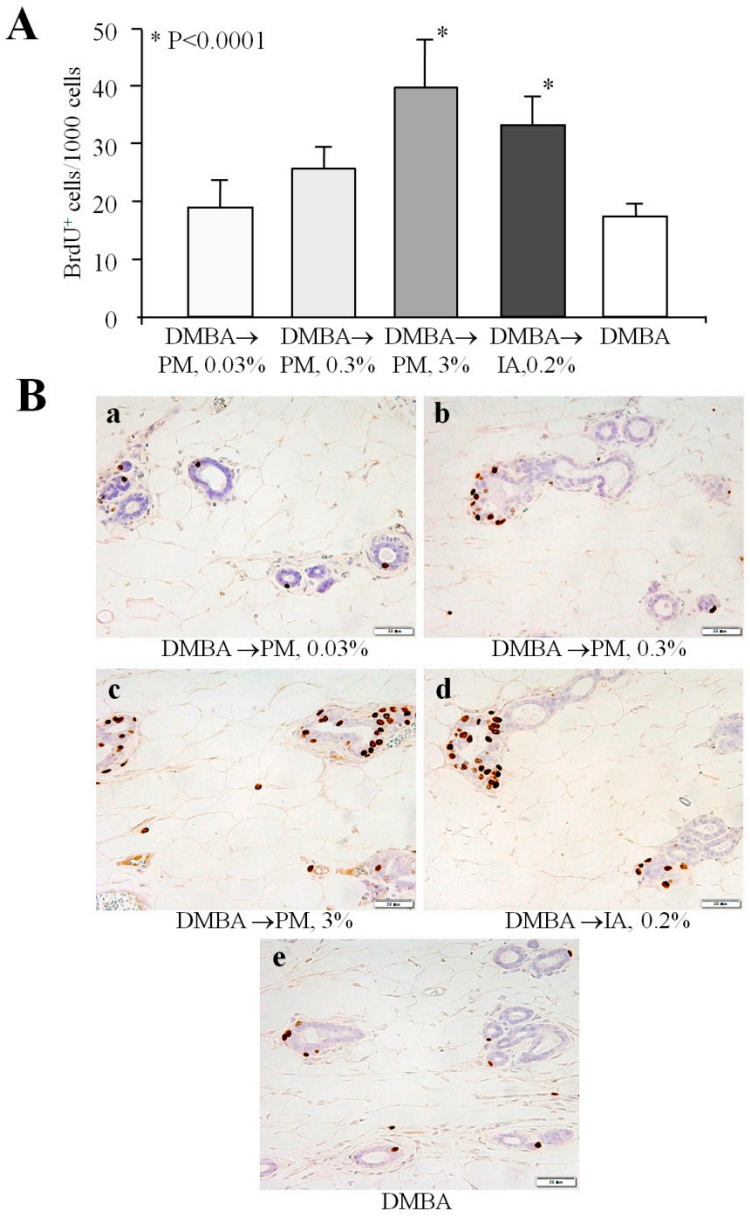

Fig. 2. BrdU labeling indices in the mammary gland of rats administered PM and IA after the DMBA initiation (A). Representative pictures of BrdU immunohistochemistry in the mammary glands of rats (B). Note the dose-dependent induction of cell proliferation by the short-term application of PM as compared to the DMBA initiation control rats.

administration (Fig. 2). The significant elevation of the number of BrdU positively

($BrdU^+$)-stained cells were detected in 3% PM ($P<0.001$) and 0.2% IA groups ($P<0.001$),

as compared to the DMBA control group.

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Long-term study (exp. 3).

Body, organ weights, food and water consumption. The rat body weight curves are presented in Fig. 3A. Body weights of 0.3% PM, 1% PM and 0.2% IA-administered rats were lower than in the initiation control group, with significant differences detected at the termination of the experiment. No changes of food intakes were observed between groups. The decreases of water consumption were found in PM and IA-treated animals.

Significant increases of relative uterus weight with 0.3 and 1% PM, and a trend for increase in the vehicle 1% PM group were found as compared to the respective control groups (Table 1). Relative liver weights were significantly decreased in 0.3 and 1% PM-treated rats. Moreover, significant elevation of relative kidneys weight was found in the 1% PM group. In addition, the significant increase of relative thymus weight at 0.03% PM and elevation of relative adrenals weight in the vehicle 1% PM group as compared to the respective controls were detected (Table 1).

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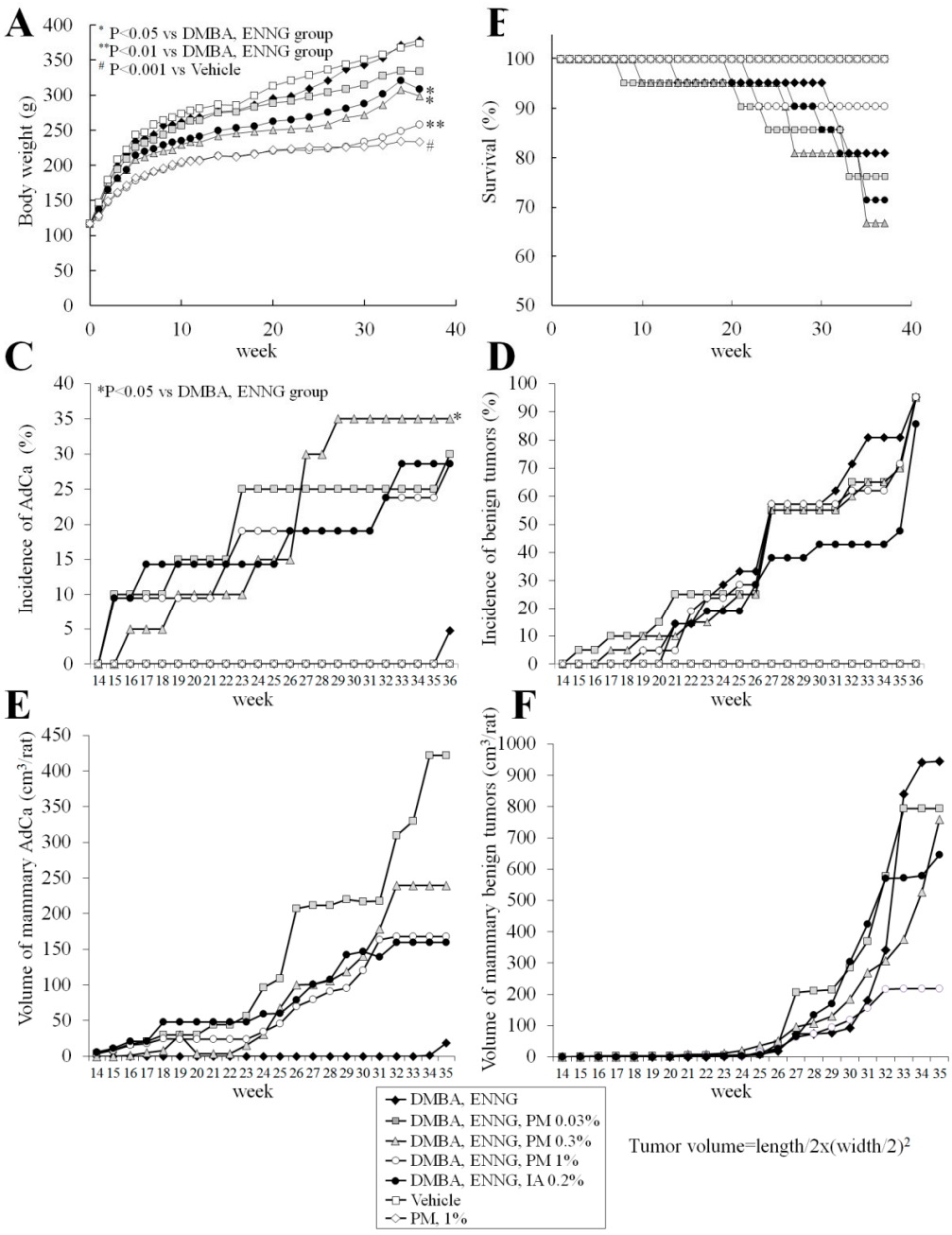

Fig. 3. Body weight (A) and survival (B) curves of female Donryu rats in experiment 3; Incidences of mammary adenocarcinoma (C), benign tumors (D), and volumes of mammary adenocarcinoma (E) and benign tumors (F).Note the significant decreases of body weights in the 0.3, 1% PM and 0.2% IA-treated Donryu rats. Trends for decrease in survival were found for 0.03, 0.3% PM and 0.2% IA groups. Significant increases of mammary adenocarcinoma incidence were observed in 0.3% PM and 0.2% IA-treated rats. Adenocarcinomas in PM and IA-treated rats appeared earlier, and their volumes were higher than in the initiation control group. Sudden development of benign tumors in the initiation control group starting at week 32 was obvious. Mammary tumors were larger in animals with higher body weights.

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Table 1. Final body, relative organ weights and chemicals intakes of Donryu rats in experiment 3.

Treament	No. rats[a]	Final body weight (g)	Food consumption (g/rat/day)	Chemical intake (mg/rat/day)	Chemical intake (mg/kg b.w./day)	Total chemical intake (g/rat)
DMBA, ENNG	21	375.5±89.4	17.5±3.6	0±0	0±0	0±0
DMBA, ENNG→ PM, 0.03%	21	344.1±77.6	17.5±5.8	5±2	21±13	1.4
DMBA, ENNG→ PM, 0.3%	21	306.8±82.1*	19.2±8.4	58±25	263±176	14.9
DMBA, ENNG→ PM 1%	21	263.3±49.0***	17.9±2.5	179±25	814±171	45.1
DMBA, ENNG→ IA, 0.2%	21	323.8±65.7	18.2±4.9	36±10	160±95	9.4
Vehicle	5	372.1±42.9	19.6±3.4	0±0	0±0	0±0
Vehicle→PM, 1%	6	237.3±30.9[b]	15.4±3.6	159±23	728±104	40.1

Treatment	No. rats	Uterus (%)	Liver (%)	Kidneys (%)	Spleen (%)	Thymus (%)	Adrenals (%)
DMBA, ENNG	21	0.33±0.03	4.20±1.92	0.64±0.17	0.51±1.37	0.075±0.091	0.026±0.023
DMBA, ENNG→ PM, 0.03%	21	0.41±0.07	3.70±0.79	0.64±0.11	0.34±0.28	0.186±0.144**	0.022±0.006
DMBA, ENNG→ PM, 0.3%	21	0.47±0.06*	3.84±0.74*	0.73±0.14	0.22±0.06	0.046±0.021	0.024±0.007
DMBA, ENNG→ PM 1%	21	1.35±0.52*	3.73± 0.60***	0.79±0.14***	0.37±0.48	0.048±0.018	0.031±0.016
DMBA, ENNG→ IA, 0.2%	21	0.36±0.04	3.82±1.51	0.66±0.10	0.35±0.31	0.054±0.019	0.023±0.005
Vehicle	5	0.27±0.02	2.66±0.26	0.58±0.05	0.17±0.03	0.063±0.022	0.016±0.003
Vehicle→PM, 1%	6	0.99±0.71	3.20±0.16	0.81±0.07#	0.19±0.03	0.065±0.014	0.022±0.003##

*P<0.05; **P<0.01; ***P<0.001 vs DMBA, ENNG group; #P<0.05; ##P<0.01 vs vehicle control group

Survival. The changes in rat survival are shown in Fig. 3B. Four animals in the initiation control, 6 rats in 0.03% PM, 7 rats in 0.3% PM, 2 rats in 1% PM and 6 rats in 0.2% IA groups died during the study. One rat in the vehicle control, 0.03% PM and 0.3% PM group each were found dead with no discernible cause. The causes of death in the initiation control group were zymbal gland tumor (1 rat) and malignant lymphoma/leukemia (3 rats). On the contrary, causes of death in 0.03%, 0.3%, 1% PM and 0.2% IA groups were mammary adenocarcinomas (0.03% PM: 2 rats; 0.3% PM: 2 rats; 1% PM: 1 rat; IA: 3 rats), bleeding from big necrotic mammary tumors (0.03% PM:2 rats; 0.3% PM: 1 rat; 1% PM: 1 rat), lymphoma-leukemia (0.03% PM:2 rats; 0.3% PM: 1 rat; IA: 2 rats), thymoma (0.3% PM: 1 rat) and uterine carcinoma (IA:1 rat). Two rats in 0.03% PM group, 1 rat in 0.3% PM and 3 animals in IA-treated groups

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featured metastasis of mammary adenocarcinomas in the lung.

The first rat was found dead at week 9, which was from 0.03% PM group, and the cause of death was lymphoma/leukemia. Then, one rat in 0.3% PM group at week 10 and one rat from the initiation control group at week 15 died from lymphoma/leukemia. Next, the number of animals in 0.03, 0.3% PM and IA-treated groups, but not 1% PM group, started to decrease mostly due to the development of mammary adenocarcinomas (Fig. 3B). Survival rates of 0.03, 0.3% PM and IA-administered rats showed a trend for decrease at the termination of the experiment, hence without significance.

Histopathological analysis of the mammary gland. The data for the changes of mammary gland adenocarcinoma and benign tumors incidences, multiplicities, volumes, as well as histopathological analysis and representative pictures are shown in Fig.3C-F, Fig. 4A (c-d) and Table 2.

Table 2. Incidence and multiplicity of neoplastic lesions in the mammary glands and uterus of Donryu rats.

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Values are mean±SD; [a]Effective number of rats; [*]Significantly different from the DMBA, ENNG control group at $P<0.05$; [b, c, d]Significantly different from the Vehicle control group at $P<0.05$, $P<0.01$ and $P<0.0001$.

Treatment	No. rats[a]	Fibroadenoma	Fibroma	Adenoma	AdCa
Mammary gland					
Incidence (no. rats (%))					
DMBA, ENNG	21	20(95.2)	7(33.3)	3(14.3)	1(4.8)
DMBA, ENNG→ PM, 0.03%	20	19(95)	8(40)	2(10)	6(30)
DMBA, ENNG→ PM, 0.3%	20	19(95)	8(40)	4(20)	7(35)[*]
DMBA, ENNG→ PM 1%	21	20(95.2)	5(23.8)	4(19.1)	6(28.6)
DMBA, ENNG→ IA, 0.2%	21	18(85.7)	3(14.3)	2(9.5)	6(28.6)
Vehicle	5	1(20)	0	0	0
Vehicle→PM, 1%	6	1(16.7)	0	0	0
Multiplicity(no./rat)					
DMBA, ENNG	21	10.90±4.93[d]	0.55±0.89[b]	0.15±0.37	0.05±0.22
DMBA, ENNG→ PM, 0.03%	20	9.85±5.39	0.40±0.50	0.10±0.31	0.40±0.68
DMBA, ENNG→ PM, 0.3%	20	8.30±4.93	0.50±0.69	0.20±0.41	0.45±0.69[*]
DMBA, ENNG→ PM 1%	21	7.95±4.26	0.29±0.56	0.19±0.40	0.33±0.58
DMBA, ENNG→ IA, 0.2%	21	6.90±5.02[*]	0.14±0.36	0.10±0.30	0.43±0.75[*]
Vehicle	5	0.20±0.45	0	0	0
Vehicle→PM, 1%	6	0.17±0.41	0	0	0

		Dilatation	Endometrial HPL				AdCa	Polyps	
Uterus			Mild	Moderate	Severe	Total		S	A
Incidence (no. rats (%))									
DMBA, ENNG	21	8(38.1)	10(47.6)	5(23.8)	2(9.5)	17(81.0)	1(4.8)	6(28.6)	3(14.3)
DMBA, ENNG→ PM, 0.03%	20	13(65)	16(80)	4(20)	1(5)	17(85)	3(15)	5(25)	2(10)
DMBA, ENNG→ PM, 0.3%	20	19(95)[**]	17(85)	7(35)	1(5)	19(95)	2(10)	11(55)	8(40)
DMBA, ENNG→ PM 1%	21	19(90.5)[**]	11(52.4)	2(9.5)	2(9.53)	12(57.1)	0(0)	4(19.0)	5(23.8)
DMBA, ENNG→ IA, 0.2%	21	19(90.5)[**]	16(76.2)	5(23.8)	4(19.0)	20(95.2)	2(9.5)	9(42.9)	9(42.9)
Vehicle	5	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Vehicle→PM, 1%	6	6(100)[*]	3(50.0)	0(0)	0(0)	3(50.0)	0(0)	0(0)	0(0)
Multiplicity (no./rat)									
DMBA, ENNG	21	-	0.67±0.80[c]	0.24±0.44	0.14±0.48	1.10±0.64[d]	0.05±0.22	0.33±0.58	0.14±0.36
DMBA, ENNG→ PM, 0.03%	20	-	1.25±0.91[*]	0.24±0.54	0.05±0.22	1.55±1.00	0.15±0.37	0.25±0.44	0.15±0.47
DMBA, ENNG→ PM, 0.3%	20	-	1.35±0.81[*]	0.35±0.49	0.05±0.22	1.75±0.79[*]	0.10±0.31	0.95±1.19	0.65±1.09
DMBA, ENNG→ PM 1%	21	-	0.57±0.60	0.10±0.30	0.14±0.48	0.81±0.81	0.00±0.00	0.43±1.33	0.33±0.73
DMBA, ENNG→ IA, 0.2%	21	-	1.00±0.71	0.24±0.44	0.19±0.40	1.52±0.60[*]	0.10±0.30	0.57±0.75	0.57±0.75[*]
Vehicle	5	-	0	0	0	0	0	0	0
Vehicle→PM, 1%	6	-	0.50±0.55	0	0	0.50±0.55	0	0	0

AdCa, adenocarcinoma; HPL, hyperplasia; S, stromal polyp; A, adenomatous polyp.

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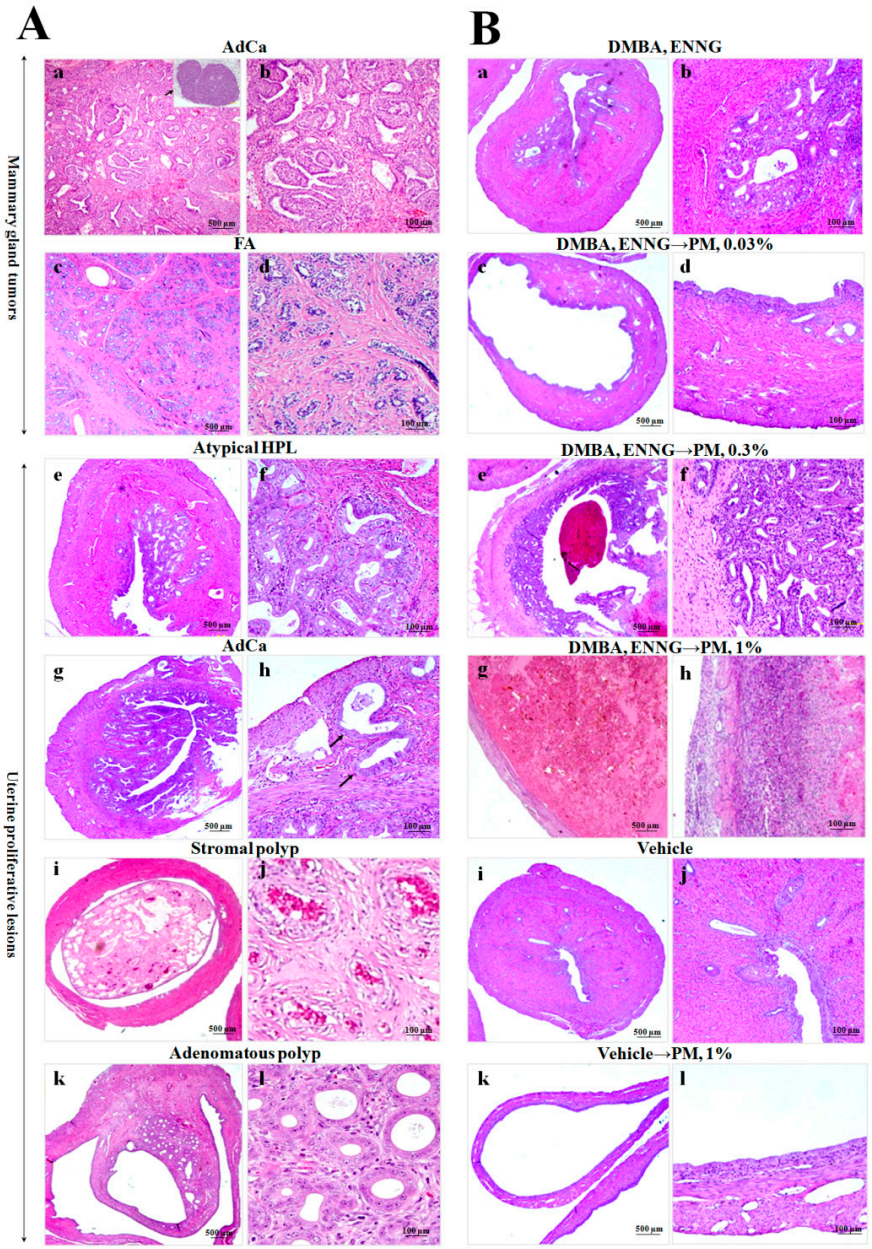

Fig. 4. (A) Histopathological changes observed in the mammary gland (a-d) and uteri (e-l) of initiation control, PM and IA-administered rats in experiment 3 (H & E). Moderate atypical hyperplasia composed of an increased number of glands under the lining epithelium (e, f). Well-differentiated endometrial adenocarcinoma (AdCa) (g, h). Atypical glands present in the endometrium proliferate irregularly and invade the muscle layer (arrows). Stromal polyp (i, g) and adenomatous polyp (k, l) (B) Histopathological changes in the uteri of the DMBA, ENNG control (a, b); 0.03 (c, d), 0.3 (e, f) and 1% (g, h) PM-treated Donryu rats after initiation; vehicle control (i, j) and vehicle 1% PM (k, l)-administered rats in the experiment 3. Note the development of atypical hyperplasia (DMBA, ENNG→ 0.3% PM group), uterus dilatation (0.3 and 1% PM-treated rats), inflammation and hemorrhage (DMBA, ENNG→ 0.3 and 1% PM groups) induced by the PM treatment. (Magnifications in (A) and (B): ×20 (a, c, e, g, i, k) and ×200 (b, d, f, h, j, l)).

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Results of histopathological analysis demonstrated that incidences and/or multiplicities of mammary adenocarcinoma were significantly elevated in 0.3% PM and IA-administered rats ($P<0.05$) (Fig.3C and Table 2). On the contrary, dose-dependent non-significant decreases of fibroadenoma and total benign mammary tumor incidences and multiplicities in groups fed PM, and significant inhibition in IA-treated rats ($P<0.05$) was found (Fig. 3D and Table 2).

Macroscopically measured mammary adenocarcinoma volumes were elevated in 0.03, 0.3, 1% PM and 0.2% IA groups starting from week 16 as compared to the initiation control rats, with the highest value observed in the 0.03% PM dose group and the lowest elevation induced by PM at a dose of 1% (Fig. 3E). Unexpectedly, sudden development of benign tumors in the mammary gland of the initiation control rats was observed at weeks 32-36. Animals with higher body weights had larger mammary tumors (Fig. 3F).

Histopathological analysis of the uterus. The data on the histopathological examination of rat uteri are shown in Table 2 and Fig. 4A (e-l) and 4B (a-l). At termination, the uteri of 0.3, 1% PM as well as IA-treated rats after initiation of endometrial carcinogenesis demonstrated dilatation, increased hemorrhage, and higher numbers of nodules macroscopically. In the uteri of ENNG-initiated rats, we observed various proliferative lesions, with a sequence of changes from atypical hyperplasias to adenocarcinomas (Fig. 4A (e-h), furthermore, stromal and adenomatous polyps were obvious (Fig. 4A (i-l). A significant increase in the multiplicities of total atypical hyperplasia (HPL) (mild, medium and severe) was found in 0.3% PM and IA-administered rats (Table 2). Furthermore, significant elevation of multiplicity of mild atypical HPL was detected in the uteri of 0.03 and 0.3 % PM groups. Trends for increase of incidences and multiplicities of uterus adenocarcinomas in 0.03 and 0.3% PM and 0.2% IA, but not the 1% PM-treated rats were also found. In addition, multiplicities of stromal and adenomatous polyps tended to be increased by 0.3% and 1% PM treatment, while the significant elevation was detected in the uteri of the IA-administered rats (Table 2).

Blood hematology and biochemistry. The results of hematological and biochemical examinations of the blood are presented in Table 3.

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Table 3. Results of the hematological and blood biochemical analyses.
Values are means ± SD; *P < 0.05;**P<0.01; ***P<0.001 vs DMBA, ENNG initiation control group;

	DMBA, ENNG					Vehicle	
Test compound	PM				IA	Vehicle	PM
Dose of test compound (%)	0	0.03	0.3	1	0.2	0	1
No. of animals examined	17	15	13	19	15	5	6
WBC (/μl)	4550±1777	6479±8488	4525±2235	4141±2463	7547±12949	3600±1706	3320±996
RBC (x10^4/μl)	593.0±95.6$^{\#}$	589.4±112.3	635.1±125.7	612.7±113.8	625.9±185.1	770.4±32.6	728.6±31.9
Hb (g/dl)	11.7±1.6$^{\#}$	11.6±2.6	11.94±2.0	11.6±2.0	11.8±3.4	14.1±0.6	13.9±1.1
Ht (%)	38.0±4.7$^{\#}$	35.7±7.3	37.9±5.8	36.6±6.0	37.6±10.1	44.7±0.9	42.1±1.8$^{\#}$
MCV (fl)	64.6±4.2	60.6 ±5.0	60.4 ±4.6	60.1±3.3	62.3±8.2	58.2±1.8	57.8±0.8
MCH (pg)	19.9±1.0	19.9±5.7	18.9±1.0	19.0±0.8	19.3±2.4	18.4±0.8	19.1±1.1
MCHC (g/dL)	30.8±0.8	32.8±8.0	31.4±1.2	31.7±0.9	31.1±1.9	31.6±0.9	32.9±2.0
Platelets (x10^{10}/L)	76.7±13.0$^{\#\#}$	57.4±24.2*	39.1±31.9**	54.2±25.5**	56.2±28.5	38.5±20.0	56.4±15.8
Neutrophils (x10^3/L)	32.2±17.0	42.1±23.9	25.8±15.1	34.3±14.4	31.9±15.7	30.0±5.6	42.8±8.1$^{\#}$
Band neutrophils (x10^3/L)	1.4±0.6	1.1±0.4	1.2±0.4	1.1±0.2	1.4±0.9	1.0±0.0	1.0±0.0
Eosinophils (x10^3/L)	1.2±1.2	0.9±1.2	0.7±1.0	0.7±1.6	0.7 ±1.3	1.0±0.7	0.6±0.5
Basophils (x10^3/L)	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
Monocytes (x10^3/L)	2.9±1.4	2.4±1.5	3.5±1.3	3.9±2.3	2.8±1.8	3.0±1.6	3.8±1.5
Lymphocytes (x10^3/L)	62.3±16.7	53.5±24.0	68.8±15.8	60.1±14.0	62.7±16.2	65.0±6.7	51.6±9.8$^{\#}$
T-protein (g/dl)	7.3±1.0	6.7±0.6	7.1±0.5	6.8±0.8	7.2±0.4	7.5±0.4	7.6±0.5
Albumin (g/dL)	5.4±0.5	4.8±0.8	5.2±0.6	4.9±0.8	5.3±0.5	5.1±0.4	5.4±0.4
A/G ratio	3.2±0.9	2.6±0.8	3.0±0.9	2.6±0.7	3.0±0.7	2.1±0.4	2.6±0.5
T-BiL (mg/dL)	0.10±0.00	0.10±0.00	0.10±0.00	0.11±0.02	0.11±0.03	0.14±0.1	0.10±0.00
AST (IU/L)	428.1±216.9	425.1±528.6	378.2±295.2	263.7±132.6*	375.2±296.6	467.0±105.7	223.2±19.4$^{\#\#}$
ALT (IU/L)	62.7±39.8	62.8±77.6	87.4±84.0	55.6±28.8	88.8±89.1	96.2±41.1	55.7±12.0$^{\#}$
ALP (IU/L)	62.0±34.0	147.7±253.6	73.6±18.3	99.7±58.6*	77.5±38.3	163.9±98.1	125.3±51.0
γ-GTP (IU/L)	2.1±3.1	3.0±2.9	2.5±1.6	5.5±11.7**	3.9±2.9**	1.0±0.0	2.2±1.0$^{\#}$
T-Cholesterol (mg/dL)	141.5±35.4$^{\#}$	123.5±48.2	123.0±30.0	105.3±27.9*	130.0±36.9	191.6±39.0	116.7±31.8$^{\#\#}$
TG (mg/dL)	90.6±51.5$^{\#}$	76.7±44.7	65.5±60.2	68.1±46.3	111.9±169.2	47.4±19.2	39.7±28.4
BUN (mg/dL)	22.3±4.1$^{\#}$	25.1±7.2	24.2±4.0	27.1±9.9	22.0±7.0	17.2±2.2	23.7±4.4$^{\#}$
Creatinine (mg/dL)	0.31±0.07	0.33±0.05	0.35±0.06	0.29±0.08	0.29±0.04	0.37±0.05	0.36±0.03
Na (mEq/L)	143.9±1.4	143.9±1.3	143.0±2.7	143.4±1.8	143.4±1.8	143.6±1.9	144.7±1.6
K (mEq/L)	5.0±0.9	5.1±0.5	5.6±3.7	4.8±0.9	4.9±0.7	4.8±0.3	4.6±0.4
Cl (mEq/L)	100.1±1.7	100.5±1.7	99.8±1.6	100.2±2.0	100.5±2.1	98.4±1.9	99.2±1.8
Ca (mEq/L)	9.9±0.4	9.7±0.4	9.7±0.4	9.4±0.4***	9.6±0.3	10.2±0.5	9.7±0.4
IP (mEq/L)	6.8±1.6	7.2±1.4	6.3±2.5	5.9±1.2	5.5±1.7	7.9±3.2	6.4±1.1

$^{\#}$P<0.05; $^{\#\#}$P<0.01 vs vehicle control group.
TG, triglycerides; T-Bil, T-bilirubin; IP, inorganic phosphorus.

The significantly decreased red blood cell count, Hb and Ht, but increased platelet count was observed in DMBA and ENNG-initiated rats as compared to the vehicle control rats. When the PM at a dose of 1% was administered without the initiation, Ht and lymphocyte count were significantly suppressed but neutrophils elevated indicating higher levels of inflammation. Furthermore, PM administration after the initiation of mammary and uterine carcinogenesis significantly and dose-dependently suppressed the platelet counts.

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In blood biochemistry, significant inhibition and a trend for decrease in total cholesterol and triglyceride levels were found in PM and IA-administered rats after initiation and in the vehicle 1% PM group. Moreover, in 1% PM and IA-treated rats, the AST and ALT were suppressed, but the ALP and γ -GTP elevated. Furthermore, in the initiation control and 1% PM vehicle groups, the blood BUN level was increased as compared to the vehicle control group. In addition, a significant inhibition and trends for decrease of blood calcium levels were found in 1% PM and other PM groups, respectively, as compared to the respective controls.

Discussion

The present results demonstrated that the long-term postpubertal exposure to PM at doses higher than 200 mg/kg b.w/day possessed estrogenic activity and induced cell proliferation in the mammary gland of Donryu rats. Furthermore, the long-term treatment with PM at 200 mg/kg b.w/day promoted mammary and endometrial carcinogenesis after DMBA and ENNG initiation. Mammary adenocarcinomas metastasizing to the lungs were found in 0.03 and 0.3% PM, as well as 0.2% IA-treated rats. In this study, the modifying effect of 0.3% PM on mammary gland and uterus was comparable with that of 0.2% IA. In the medium and especially high dose PM groups, decreases of rat body weights, adipose deposition, and total cholesterol and triglycerides levels in the blood were obvious, which was likely due to antilipogenic effects of estrogenic compounds described previously, or due to the decrease of water intakes of rats by PM [16]. In the long-term experiment, we observed the significant increase of mammary adenocarcinoma development induced by PM at a dose of 0.3%. The absence of dose-dependency in the effect of PM on mammary adenocarcinoma, may be related to the side effects exerted at high doses, or to the best incorporation of its ingredients reaching the working "physiological" intracellular concentration at a dose of 0.3%.

Interestingly, short and long-term administration of PM applied at medium and high dose resulted in increase of uterus weight, dilatation, hemorrhage and inflammation of the uterine wall. The effect of PM on the uterus was much less pronounced as compared to the mammary gland, and the atypical hyperplasia was elevated only at 0.3% PM.

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Trophic effects of estrogenic compounds on mammary gland and uterus were previously suggested to be due to activation of estrogen receptors ERα and ERβ signaling [17]. It was reported that PM phytoestrogens at high dose could effectively outcompete 17 β-estradiol binding to the ERα in MCF-7 cells [3]. We have previously shown that IA at an estrogenic dose of 150 mg/kg b.w./day activated ERα or ERβ and downstream AP1 and NF-kB transcriptional factors, furthermore, potentiated F-actin signaling in mammary and uterine adenocarcinomas [15]. However, the effects of biological substances possessing estrogenic activity appeared to be dependent on the dose of their exposure. In case of IA intake the ”physiological” concentrations are known as those achieved in the serum of persons consuming the commonly recommended daily dose of isoflavones of 50-100 mg [18]. However, in case of PM, there is almost no data, concerning the concentration of PM compounds in the blood and tissues. The safe dose for human is considered as 1-2 mg/kg b.w./day. From our results, the dose of PM exerting promoting effects on mammary and uterine carcinogenesis in rats is close to 200 mg/kg b.w./day (0.3%).

In female monkeys, daily treatment with 100 mg and 1,000 mg/day (about 20 and 200 mg/kg b.w./day) of PM for 90 days produced a dose-dependent reduction in the urinary follicular stimulating hormone (FSH), luteinizing hormone (LH) and estradiol levels in the blood, and the single dose of 1,000 mg disturbed the ovarian function and menstrual cycle [19-22]. Furthermore, recent experiments in mice demonstrated that the oral exposure to non-toxic PM dose of 100 mg/kg b.w./day for 8 weeks, resulted in the prolonged estrous cycles, while the 10 mg/kg b.w./day does not induce any changes in the hypothalamic-pituitary-ovarian-uterine axis, and did not exert an estrogenic activity or the adverse effects on the mating efficiency or reproduction of mice [23]. In addition, the development of the uterine endometrial hyperplasia and a decrease in the number of growing ovarian follicles, possibly related to the reductions in the LH and FSH levels, were detected after PM application to mice at a dose of 100 mg/kg b.w./day but not the 10 mg/kg b.w./day. Moreover, in studies with gonadectomized male and female rats, oral treatment with water suspended PM at doses of 100 and 1,000 mg/kg b.w./day for 2 weeks resulted in significant increase of uterine weight, remarkable vaginal and uterine proliferation, vaginal cornification and suppression of the reproductive functions, and

this response was higher in females [24-28]. The recovery after the cessation of the treatment was dependent on dosages of PM.

In support of the previous results in rats and mice, in the short-term study we observed the estrogenic effect of PM applied to ovariectomized rats at doses of 200 and 2,000 mg/kg b.w./day for 2 weeks. The long-term PM administration at a dose of 0.3% (200 mg/kg b.w./day) was found not only to exert estrogenic activity in the mammary gland and uterus, but also to promote mammary carcinogenesis and induce atypical hyperplasia in the uteri of Donryu rats.

It is important further to mention, that the timing of exposure to substances with estrogenic bioactivities is thought to be a critical component for an effect on breast cancer risk. Thus, the prepubertal and postpubertal exposure of estrogenic compounds such as genistein, could have different effects on the cell proliferation of the terminal ductul structure in the mammary gland [29]. From the present results, the postpubertal exposure of PM, isoflavones or other test compounds with estrogenic activity could induce cell proliferation and promote mammary and uterine carcinogenesis in the present two-step carcinogenesis model with DMBA and ENNG initiation.

Here, we observed that 1% PM suppressed the lymphocytes and platelet counts but elevated the neutrophils levels likely being indicative of its effect on the immune system, promoting inflammation and bone marrow suppression. Moreover, 1% PM elevated the ALP and γ-GTP as well as the BUN in the blood, what could occur if the kidneys or liver are damaged. In addition, the present results demonstrated that 1% PM induced the decrease in the blood calcium levels. These data are in line with recent results demonstrating that the long-term treatment of aged menopausal monkeys with 1,000 mg/day of PM decreases serum parathyroid hormone (PTH) and calcium levels in the blood likely due to the amelioration of the bone loss by PM which is caused by estrogen deficiency [11,12].

In conclusion, in the present study long-term postpubertal treatment of Donryu rats with PM at a dose of 200 mg/kg b.w./day exerted promoting effect on mammary and endometrial carcinogenesis after the initiation with DMBA and ENNG. Furthermore, PM elevated cell proliferation in the mammary glands of DMBA-initiated

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rats, which might lead to the promotion and progression of mammary tumors to more malignant lesions. In addition, it inhibited the levels of calcium in the blood, and induced inflammation, hemorrhage and dilatation of the uterine wall in rats.

Experimental section

Chemicals. *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) was obtained from Nakalai Tesque (Kyoto, Japan); 7,12-dimethylbenz[a]anthracene (DMBA) was from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and polyethylene glycol (PEG) was from Wako Pharmaceutical, Osaka, Japan. Other chemicals were from Sigma Chemical Co. or Wako Pharmaceutical.

Test compounds. The Pueraria *mirifica* powder (Lot No.: PM490621) was provided by Pias Corporation (Osaka, Japan). Isoflavone aglycon (IA) extract (SoyAct) was from Kikkoman Corporation (Noda City, Chiba, Japan). In the present experiment, the test powder diets were prepared as followers: 0.03%, 0.3%, 1 and 3% PM diets contained 0.03, 0.3, 1 and 3% Pueraria *mirifica* powder, respectively, in NIH-07PLD powder diet (Phytoestrogen Low Diet, Oriental Yeast, Tokyo, Japan). The accuracy of doses formulation and uniformity of blending of the diets was performed by the analytical chemistry laboratories at Oriental Yeast Co., Tokyo, Japan. For the production of the IA extract the fermentation of soy was performed following by ethanol/water extraction and purification [15].

Animals. One hundred and seventeen female 4-week-old Crlj:DON（Donryu）rats (Japan SLC, Shizuoka, Japan) were obtained at 5 weeks of age and quarantined for 1 week before experiment started. Rats were kept in an animal house with a 12 h (8:00 -20:00) light/dark cycle, humidity of 50±2% and temperature of 23±1°C. Tap water and NIH-07PLD diet (Phytoestrogen Low Diet, Oriental Yeast, Tokyo, Japan) was given *ad libitum*. NIH-07PLD diet constituents were carbohydrate, crude protein, crude fiber, fat, neutral detergent fiber, ash, fatty acids, amino acids, vitamins and trace elements with no phytoestrogens. All animals were checked once daily for general behavior and signs of toxicity or moribund state. Body weights, water and food intakes were measured every week for the first 12 weeks, and thereafter every 4 weeks. During the experiment, the specific signs used to determine when the animals should be euthanized included no

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response to stimuli or the comatose condition, changes in heart rate and physical appearance, dyspnea or severe breathing problem, hypothermia, prostration, body weight loss and changes in food and water intakes. If the significant body weight loss or the water and food consumption changes were firstly detected, those animals were checked more precisely for other signs of sickness or moribund state. At euthanization, the systemic macroscopic pathological examination of liver, kidneys, spleen, adrenals, thymus, mammary glands and uterus was performed. All experimental procedures were done after the approval and according to the Guidelines of Animal Care and Use Committee of the Osaka City University Medical School.

Short-term experiment 1. We performed ovariectomy in twenty five 5-week-old female Donryu rats with normal estrous cycles. All animals were checked by vaginal cytology for the absence of estrous cycles. Two weeks after the ovariectomy, animals were given PM at doses of 0.03, 0.3, 3%, and the 0.2% isoflavone aglycone (IA) extract in basal NIH-07PLD diet, or only the control diet, for further 2 weeks. Vaginal smears stained with aqueous solution of methylene blue were used to check the estrogenic activity of the test compounds. Animal body weights were detected once a week, and the general condition was examined once a day. The weights of uteri were measured at final necropsy to determine estrogenic effects of test compounds. Mammary gland, uterus, liver, kidneys, spleen, adrenals and thymus were subjected for the histopathological analysis.

A Donryu rat with a mean body weight of 200 g was consuming PM in 15 g diet at doses of 20 (0.03%), 200 (0.3%), 667 (1%) and 2,000 (3%) mg/kg b.w/day, which are considered to be equal to about 0.2, 2.0 and 20.0 mg/kg b.w./day (10, 50 and 1000 mg/day) intake by women with mean body weight 50 kg (ADI for rats is considered 100 times of that of human as the safety factor in terms of ADI for rats is 100 (WHO)). As the ADI of PM for human is 1-2 mg/kg b.w./day, in case of this extrapolation, the 0.3% PM dose used in our experiment is equal to those accepted safe for human (2 mg/kg b.w./day). In addition, the dose for rats could be also extrapolated to a human equivalent dose by the body surface area (BSA) normalization method (mg/m^2 conversion) [30, 31], in which the multiplication of the human dose by 6.16 is used (Km human/Km animal=37/6). In case of BSA normalization, the PM doses of 20,

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200 and 2,000 mg/kg b.w/day will be equal to 3.2, 32.5 and 325 mg/kg b.w./day intake by human, and the accepted safe dose of PM is close to the 0.03%.

Because of the too strong estrogenic effect of 3% PM observed in this experiment, the dose applied in the long-term experiment was changed from 3 to 1% (667 mg/kg b.w./day), which is equal to about 7 mg/kg b.w./day (350 mg/day) dose for human, or in the case of BSA normalization, 108 mg/kg b.w./day intake by human.

Short-term experiment 2. DMBA in sesame oil at a dose of 50 mg/kg b.w. was administered by gavage (i.g.) to twenty 5-week-old female Donryu rats. Another 5 rats were given an equivalent volume of sesame oil (~0.5 ml/rat). PM at doses of 0.03, 0.3, 3% and 0.2% IA were administered in NIH-07PLD diet for 4 weeks starting from the day of DMBA injection. At euthanasia, liver, kidneys, spleen, adrenals, thymus and uterus were weighted, and sections of mammary glands were fixed in 10% phosphate-buffered formalin for the histopathological and bromodeoxyuridine (BrdU) immunohistochemical examinations.

Long-term experiment (exp.3). One hundred and seventeen 5-week-old female Donryu rats were selected and divided into seven groups. It is known that soon after weaning, about postnatal day 35, the pubertal development of Donryu rats is started. Since the onset of puberty is defined as the age (in days) at which vaginal opening occurred, rats were inspected daily for vaginal opening. At the commencement of the experiment, 5-week-old rats from PM, IA-treated and initiation control groups (21 rats/group) were given a single dose of DMBA by i.g. (50 mg/kg b.w) for the initiation of mammary carcinogenesis. On experimental days 7 and 11, ENNG (10 mg/kg b.w.) in PEG was injected via the vagina using a stainless catheter to initiate uterine carcinogenesis. Six rats each in vehicle and 1% PM vehicle groups received sesame oil by i.g. and PEG via the vagina. PM was administered to rats at doses of 0.03, 0.3 and 1%, and IA was applied for comparison at a dose of 0.2% in NIH-07PLD basal diet for 36 weeks from the commencement of the experiment. Rats in the vehicle control group received the basal diet. One of the characteristics of Donryu rats is the age-related persistent estrus followed by anovulation starting at the age of 5 months, which incidence is rising until 8 months [32]. Mammary tumors number and volume (cm^3/rat; the formula: tumor length/2 x $(width/2)^2$) were detected once weekly, and the

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location of every nodule was recorded. Malignant tumors usually metastasized to the lung, contained abscesses and ulcers and were of dark color. The histopathologic analysis was performed according to the previously published classification of mammary tumors [33].

All surviving animals were euthanized at week 36 after and the test organs including mammary glands with skin, uterus, vaginas, ovaries, liver, pituitary, adrenals and thymus were removed, weighed and fixed in 10% formalin for the histopathological analysis. Twelve specimens were obtained from each uterus in cross-section. The proliferative endometrial lesions were classified using the categories reported previously [34] into three degrees (mild, moderate and severe) of atypical hyperplasia, adenocarcinoma, stromal and adenomatous polyps.

BrdU immunohistochemistry. In the short-term experiment 2, for the evaluation of cellular proliferation, the BrdU staining was performed in the rat mammary glands by the avidin-biotin-peroxidase complex (ABC) method reported previously [35]. Sections were incubated with mouse monoclonal anti-BrdU antibody (Dako Japan, Kyoto, Japan) at 1:500 dilution. Immunoreactivity was detected using a Vectastain Elite ABC Kit (PK-6102; Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine hydrochloride (Sigma Chemical Co., St Louis, MO, USA). A negative control was also included with staining procedure by omitting the primary antibody. At least 3,000 mammary epithelial cells nuclei were counted in each rat and the labeling indices were calculated as number of positive nuclei per 1000 cells.

Blood hematology and biochemistry. Blood samples were collected directly from the heart of all survived rats at the end of the study after overnight fasting. Automated hematology analyzer (Sysmex XE-2100, Mitsubishi Chemical Visuals, Osaka, Japan) was employed for the hematological analysis of the blood serum. The white and red blood cell counts, hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, neutrophils, band neutrophils, monocytes, basophils, eosinophils and lymphocytes counts were investigated. In the blood serum, an automatic analyzer (Olympus AJ-5200, Tokyo, Japan) was used for the biochemical analyses of total protein (T-protein, g/dL), albumin (g/dL), albumin/globulin ratio (A/G

ratio), total bilirubin (T-bil, mg/dL), aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L), alkaline phosphatase (ALP, IU/L), γ-glutamyl transpeptidase (γ-GTP, IU/L), total cholesterol (T-chol, mg/dL), triglycerides (TG, mg/dL), blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), sodium (Na), chloride (Cl), potassium (K), calcium (Ca) and inorganic phosphorus (IP) (mEq/L).

Statistical analysis. Statistical analysis was performed using the StatLight-2000(C) program (Yukms corp., Japan) or the GraphPad Prism 5 Software Inc. (CA, USA). Tumor incidences of histopathological lesions were compared by the Fisher's exact probability test or the χ^2-test, and Log rank test. Kaplan-Meier method was used for the assessment of differences in survival. The numerical data of control and experimental groups were statistically compared using the Bartlett's test. Dunnett's multiple comparison test (two-sided) was used in case of the homogeneous data, otherwise the Steel's test (two-sided) was applied. For all data P values less than 0.05 were considered significant.

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