CHAPTER IV

RESULTS

Effects of *P. mirifica* on hormone-related testicular functions, reproductive organs and fertility in male mice.

Serum LH levels

Since the levels of LH at 0 wks among those 4 groups were significantly different the LH levels in each group at 0 wks were adjusted to zero and the other data were calculated and transformed as a percent change from levels of 0 wks.

Compared to the pre-treatment levels (0 wks), serum LH levels in mice treated with DW, 10-PM and 100-PM did not change throughout the study period, except at 8 wks of DW group, the level was increased **(Figure 6)**. In contrast, serum LH levels were significantly reduced within 4 wks of DES injection, and recovered within 4 wks of the DES withdrawal.

Compared to the DW group, serum LH levels in mice treated with 10- and 100-PM did not show differences throughout the study period. Whereas, serum LH levels in mice treated with DES were significantly lower at 4-8 wks of treatment period and recovered within 4 wks after the withdrawal of DES administration.

Compared to the DES group, serum LH levels in mice treated with 10- and 100-PM were significantly higher at 4-8 wks of treatment period, and the levels were not significant different during the post-treatment period.

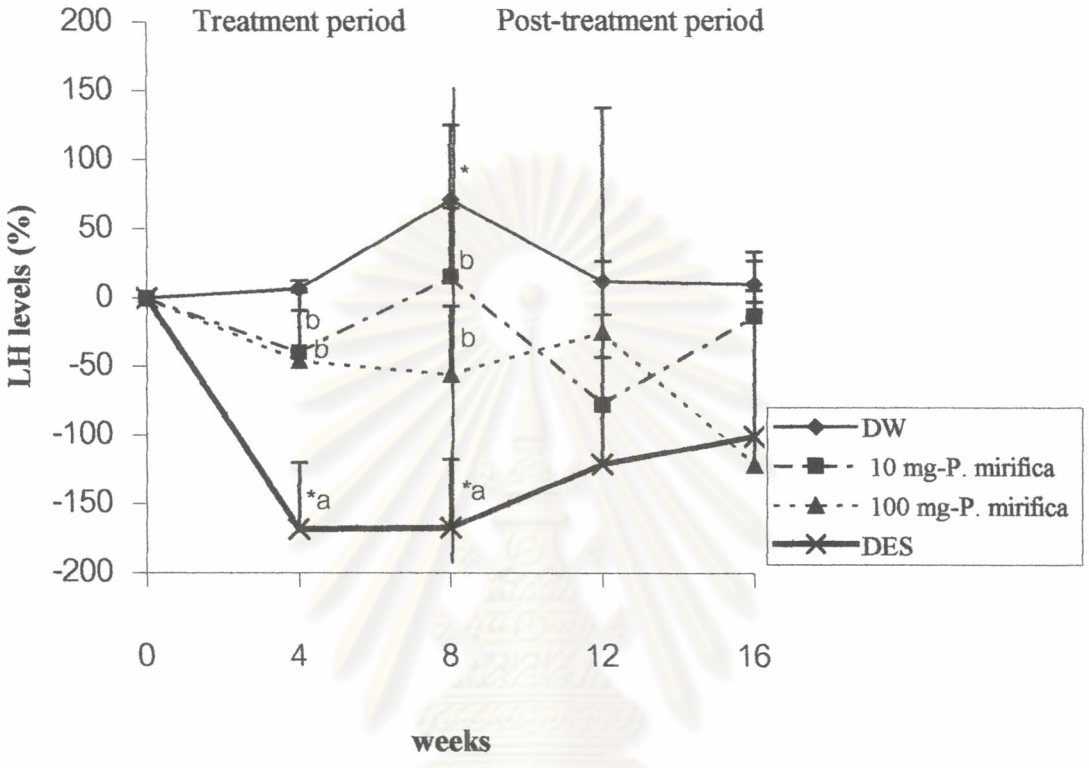


Figure 6. Percent change of serum LH levels in male mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Serum FSH levels

Compared to the pre-treatment levels (0 wks), serum FSH levels in mice treated with DW and 10-PM did not change throughout the study period **(Figure 7)**. Serum FSH levels in mice treated with 100-PM did not changes during the treatment period, and significantly decreased at 4 wks of post-treatment period. In contrast, serum FSH levels were reduced within 4 wks of DES injection, and recovered within 4-8 wks of DES withdrawal.

Compared to the DW group, serum FSH levels in mice treated with 10- and 100-PM did not show any differences throughout the study period. In contrast, serum FSH levels were lower within 4 wks after DES injection, and recovered during 4-8 wks of DES withdrawal.

Compared to the DES group, serum FSH levels in mice treated with 10- and 100-PM were significantly higher at 4-8 wks of treatment period, and the levels were not significant differences during the post-treatment period.

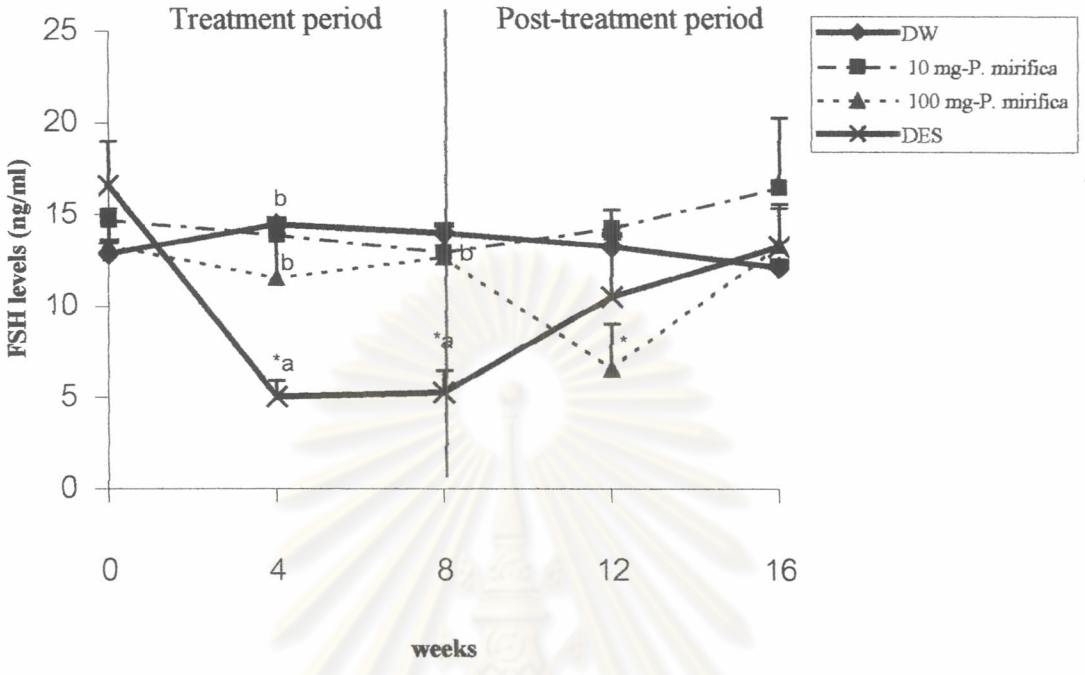


Figure 7. Serum FSH levels in male mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Serum T levels

Since the levels of T at 0 wks among those 4 groups were significantly different the T levels in each group at 0 wks were adjusted to zero and the other data were calculated and transformed as a percent change from levels of 0 wks.

Compared to the pre-treatment levels (0 wks), serum T levels in DW, 10- and 100-PM treated group did not change throughout the study period, except at 4 and16wks of DW group the levels were increased **(Figure 8)**. In agreement with the reduction of LH and FSH levels, serum T levels were reduced at 4 and 8 wks of DES injection, and recovered within 4 wks of DES withdrawal.

Compared to the DW group, serum T levels in mice treated with 10-PM did not difference during the treatment period, but the levels were lower at 8 wks of post-treatment period. Serum T levels in mice treated with 100-PM were significantly lower at 4 wks of treatment period and 8 wks of post-treatment period. Serum T levels in DES treated group were significantly lower at 4-8 wks of treatment period and 8 wks of post-treatment period.

Compared to the DES group, serum T levels in mice treated with 10- and 100-PM were higher at 4-8 wks of treatment period, and the levels were not significant differences during the post-treatment period.

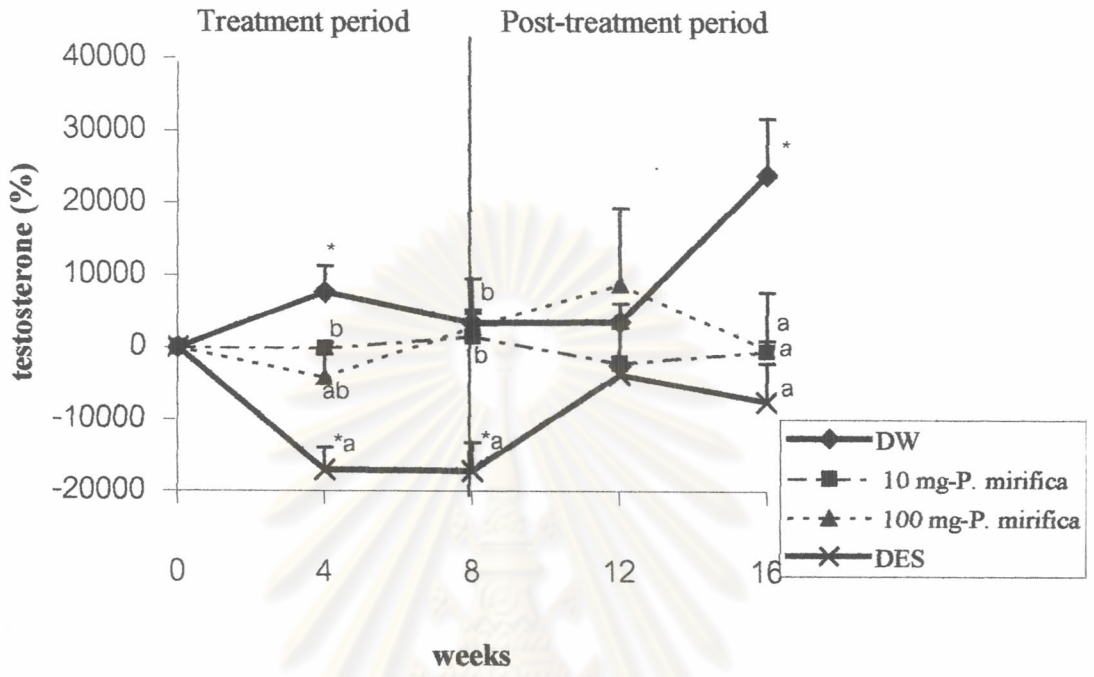


Figure 8. Percent change of serum T levels in male mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Body weights and organ weights

Compared to day-1, body weights in mice treated with DW, 10- and 100-PM did not change throughout the study period **(Figure 9)**. In contrast, body weights of DES group were significantly increased after 1 wk of injection and reached the plateau at day-15.

Compared to the DW group, body weights in mice treated with 10- and 100-PM did not difference throughout the study period. However, body weights in DES injected mice were significantly higher than the DW and both of PM treated groups since day-8 of study period.

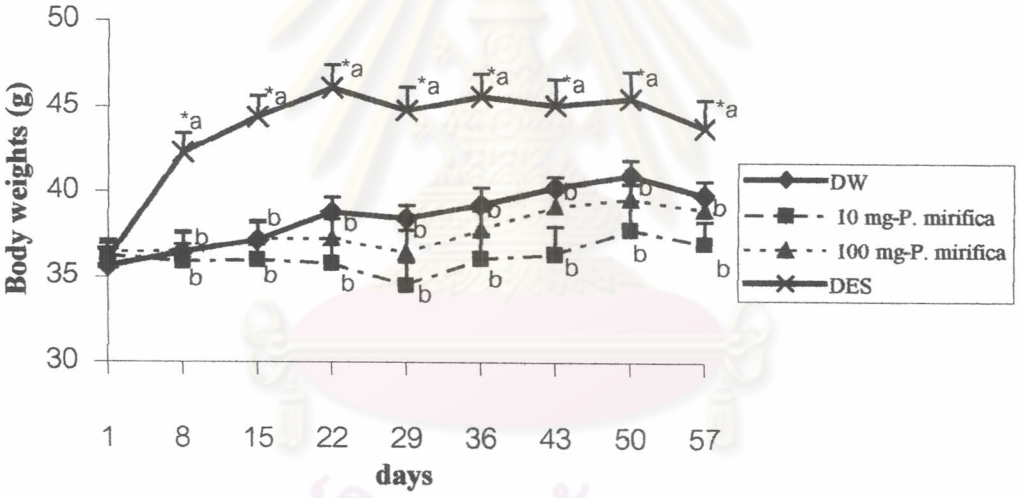


Figure 9. Body weights of male mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from day-1 ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Compared the weights of organ at the end of treatment period (K8) to the end of post-treatment period (K8P8), it was found that the seminal vesicle weights in both of *P. mirifica* treated groups and DES group were increased **(Table4)**. The epididymes weights in mice treated with 100-PM and DES at K8 were lower than K8P8, and the testes weights in DES injected mice at K8 were also lower than K8P8.

Compared to the DW group, weights of testes, epididymes, and seminal vesicle in mice treated with 10- and 100-PM did not significant differences at both K8 and K8P8, except for the weight of seminal vesicle in mice treated with 10-PM at K8P8 were higher, and the weight of epididymes and seminal vesicle in mice treated with 100-PM at K8 were lower. In contrast, weights of testes, epididymes, and seminal vesicle at K8 and only testis weights at K8P8 in mice injected with DES were lower than the DW group.

Compared to the DES group, weights of testes, epididymes, and seminal vesicle in mice treated with 10- and 100-PM were higher at K8, only testis and seminal vesicle weights in mice treated with 10-PM was significantly higher at K8P8.

Group	Testes (g)		Epididymes (g)		Seminal vesicle (g)	
	K8	K8P8	K8	K8P8	K8	K8P8
DW	0.293±0.016	0.308±0.002	0.129±0.006	0.141±0.012	0.247±0.029	0.276±0.082
10-PM	0.275±0.020[b]	0.293±0.023[b]	0.131±0.008[b]	0.1186±0.007	0.218±0.022[b]	0.440±0.029[*ab]
100-PM	0.285±0.003[b]	0.222±0.033	0.108±0.003[ab]	0.173±0.022[*]	0.173±0.010[ab]	0.2374±0.039[*]
DES	0.078±0.009[a]	0.1992±0.036[*a]	0.037±0.004[a]	0.162±0.033[c]	0.037±0.007[a]	0.201±0.013[*]

Table 4. Weights of testes, epididymes, and seminal vesicle of male mice treated with distilled water, *P. mirifica* and diethylstilbestrol. K8 and K8P8 indicate that male mice were autopsies at the end of treatment and post-treatment periods, respectively.

[*] Significantly different from K8 ($p < 0.05$)

[a] Significantly different from DW group($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Compared the relative organ weights between K8 and K8P8, it was found that the relative weights of testis, epididymis and seminal vesicle in DES injected mice at K8 were significantly lower than K8P8. The relative weights of seminal vesicle in 10-PM group at K8 was also lower than K8P8, but the relative weights of testes in mice treated with 100-PM were higher **(Table 5)**.

Compared to the DW group, the relative weight of testis and epididymis at K8 and the relative weight of seminal vesicle at K8P8 in mice treated with 10-PM were higher. The relative weights of seminal vesicle in mice treated with 100-PM at K8 were lower. The relative weights of testis, epididymis, and seminal vesicle in DES injected mice were lower at K8, whereas only the relative weights of testis were lower than the DW group at K8P8.

Compared to the DES group, the relative weights of testis, epididymis, and seminal vesicle in mice treated with 10-PM were higher at both of K8 and K8P8, except the relative weights of epididymis at K8P8 did not significant differences. The relative weights of testis, epididymis, and seminal vesicle in mice treated with 100-PM at K8 were higher, but non-significant differences at K8P8.

Group	Testes (x10 $^{-2}$)		Epididymes (x10 $^{-2}$)		Seminal vesicle (x10 $^{-2}$)	
	K8	K8P8	K8	K8P8	K8	K8P8
DW	0.737±0.037	0.704±0.022	0.323±0.010	0.318±0.022	0.616±0.056	0.613±0.166
10-PM	0.837±0.047^{ab}	0.693±0.049^{b}	0.398±0.023^{ab}	0.301±0.015	0.664±0.065^{b}	1.042±0.063^{*ab}
100-PM	0.748±0.021^{b}	0.538±0.066	0.283±0.011^{b}	0.399±0.070	0.455±0.029^{ab}	0.575±0.083
DES	0.186±0.018^{a}	0.437±0.075^{*a}	0.088±0.007^{a}	0.364±0.084*	0.086±0.013^{a}	0.445±0.030*

Table 5. Relative weights of testes, epididymis, and seminal vesicle of male mice treated with distilled water, *P. mirifica* and diethylstilbestrol. K8 and K8P8 indicate that male mice were autopsies at the end of treatment and post-treatment periods, respectively.

* Significantly different from K8 ($p < 0.05$)

^{a} Significantly different from DW group ($p < 0.05$)

^{b} Significantly different from DES group ($p < 0.05$)

Characteristics of sperm and spermatogenesis

Compared between K8 and K8P8, it was found that the sperm concentration in K8 was significantly lower than K8P8 in all 4 groups, while the sperm viability and motility in K8 was lower than K8 P8 only in DES injected group **(Table 6)**.

Compared to the DW group, the sperm concentration, viability and motility in mice treated with 10-PM did not significant differences at both K8 and K8P8. The mice treated with 100-PM showed the lower sperm viability and motility at K8 and sperm concentration at K8P8 than that of DW group. Sperm concentration, viability and motility in mice injected with DES were significantly lower than the DW group in both K8 and K8P8.

Compared to the DES group, the sperm concentration in mice treated with 10- and 100-PM were higher in both K8 and K8P8, but sperm viability and motility were higher only in K8.

Group	Sperm count ($x10^6$)		Sperm viability (%)		Sperm motility (%)	
	K8	K8P8	K8	K8P8	K8	K8P8
DW	12.040±1.582	24.020±3.308[c]	60.400±3.264	73.800±5.295	58.676±2.653	59.917±1.745
10-PM	8.560±1.642[b]	19.200±3.589[bc]	62.600±1.818[b]	71.800±2.615	56.528±3.880[b]	64.393±3.786
100-PM	9.040±1.994[b]	13.440±5.754[*ab]	42.200±3.455[ab]	55.600±15.735	40.694±3.257[ab]	50.910±15.978
DES	0±0.000[a]	8.640±2.771[*a]	0±0.000[a]	43.200±11.633[*a]	0±0.000[a]	32.755±2.918[*a]

Table 6. Sperm concentration, viability and motility of male mice treated with distilled water, *P. mirifica* and diethylstilbestrol. K8 and K8P8 indicate that male mice were autopsies at the end of treatment and post-treatment periods, respectively.

[*] Significantly different from treatment period ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Histological structure of testis in DW and 10- and 100-PM groups at K8 showed numerous of spermatogenic cells in various stages such as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatid and spermatozoa **(Figure 10-12)**. In addition, abundant spermatozoa in the seminiferous tubules and more condense than the DW group were found at K8P8. In contrast, mice treated with DES showed a thin layer of spermatogenic lineage, an absence of spermatid and spermatozoa, a few spermatogenic cells, and an evidence of Ledig cells hypertrophy **(Figure 13)**. However, all these histological alterations could be partially recovered within 8 wks (K8P8) after DES withdrawal.

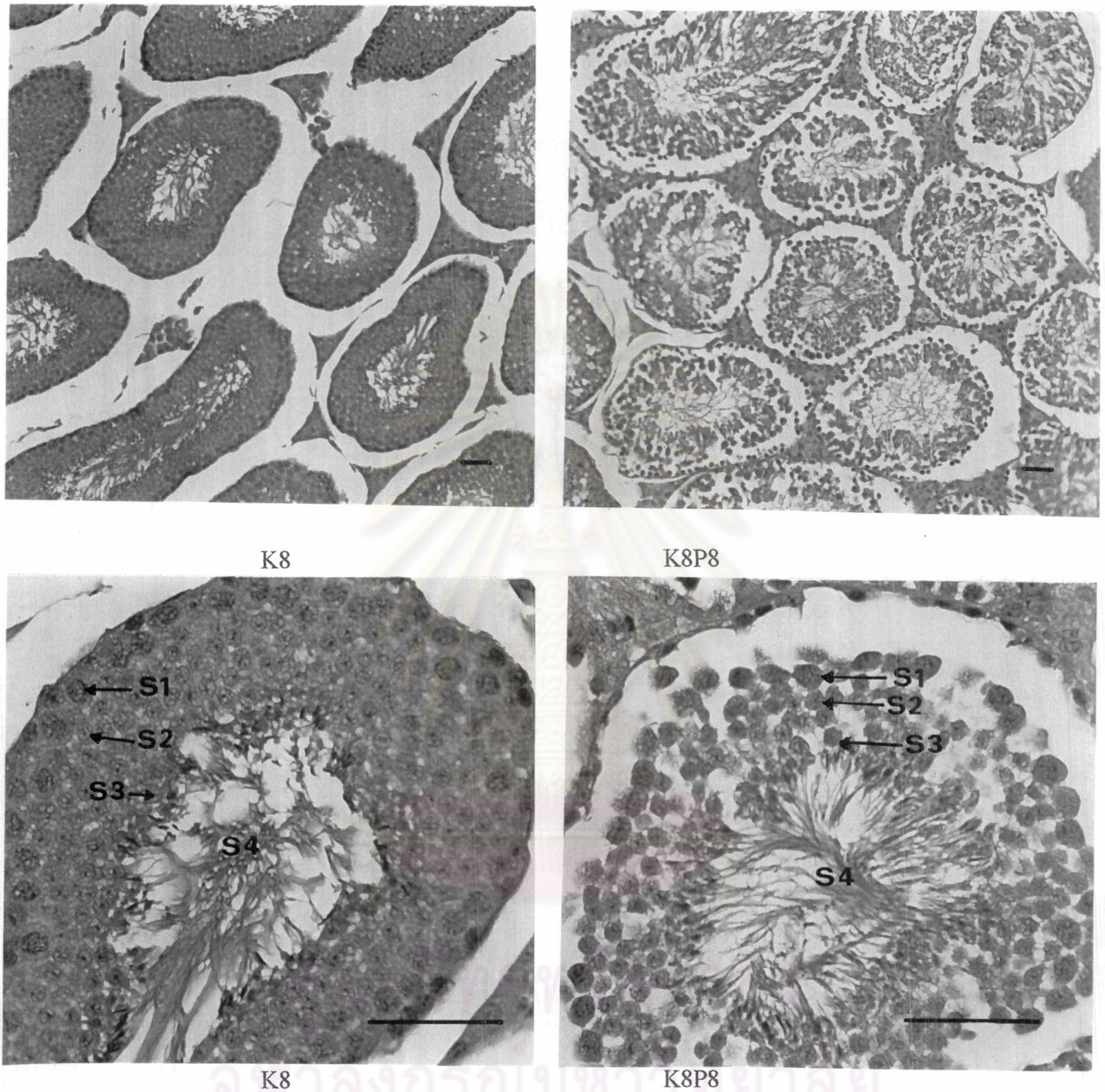


Figure 10. Testicular morphology in DW group.

L= Leydig's cells, S1= primary spermatocyte, S2= secondary spermatocyte, S3= spermatid, S4= spermatozoan. H & E stain. (Scale bars = 50 μm)

K8 and K8P8 indicate that male mice were autopsied at the end of treatment and post-treatment periods, respectively.

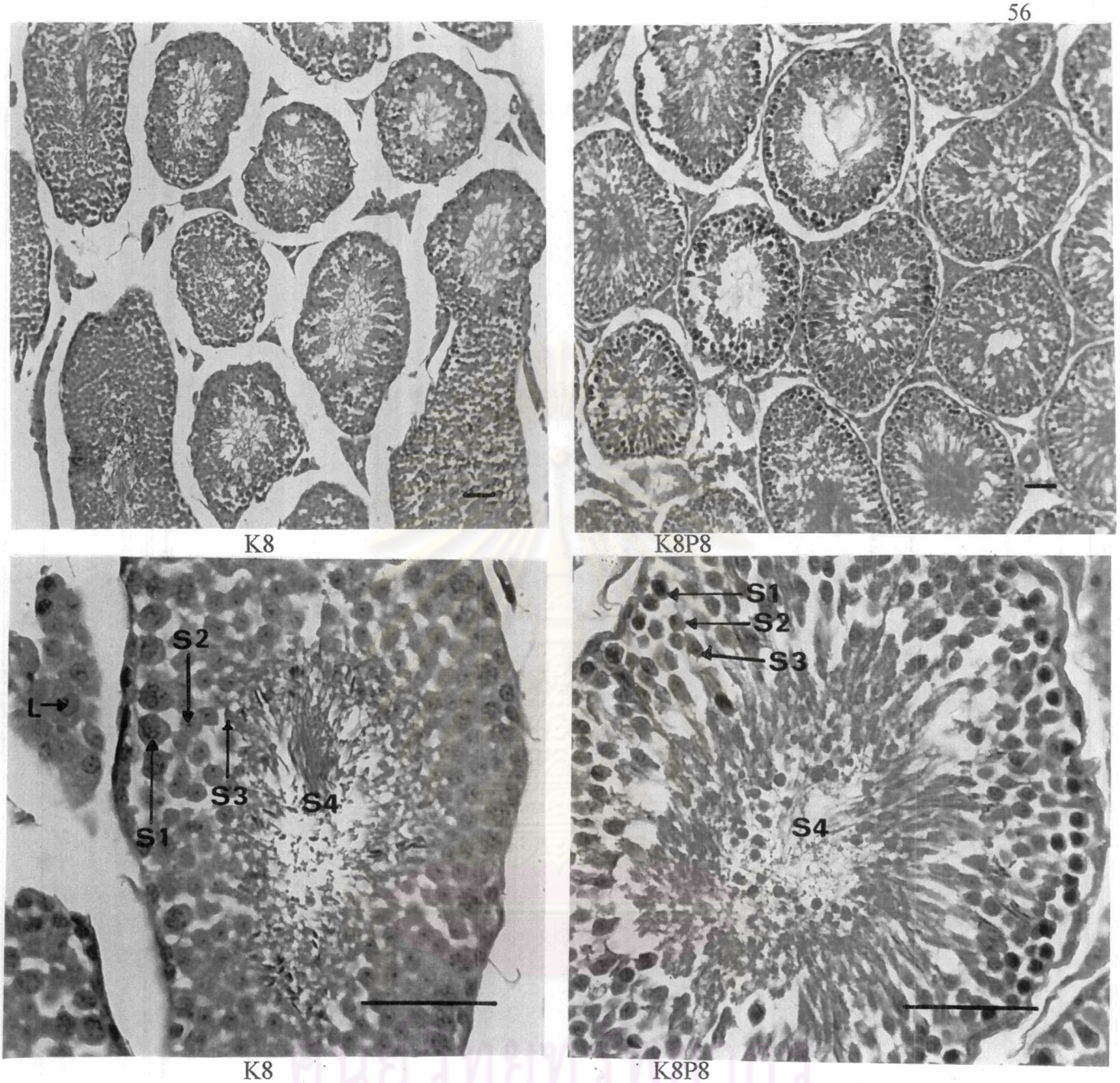


Figure 11. Testicular morphology in mice treated with 10 mg/kg BW/day of *P. mirifica*.

L= Leydig's cells, S1= primary spermatocyte, S2= secondary spermatocyte, S3= spermatid, S4= spermatozoan. H & E stain. (Scale bars = 50 μm)

K8 and K8P8 indicate that male mice were autopsied at the end of treatment and post-treatment periods, respectively.

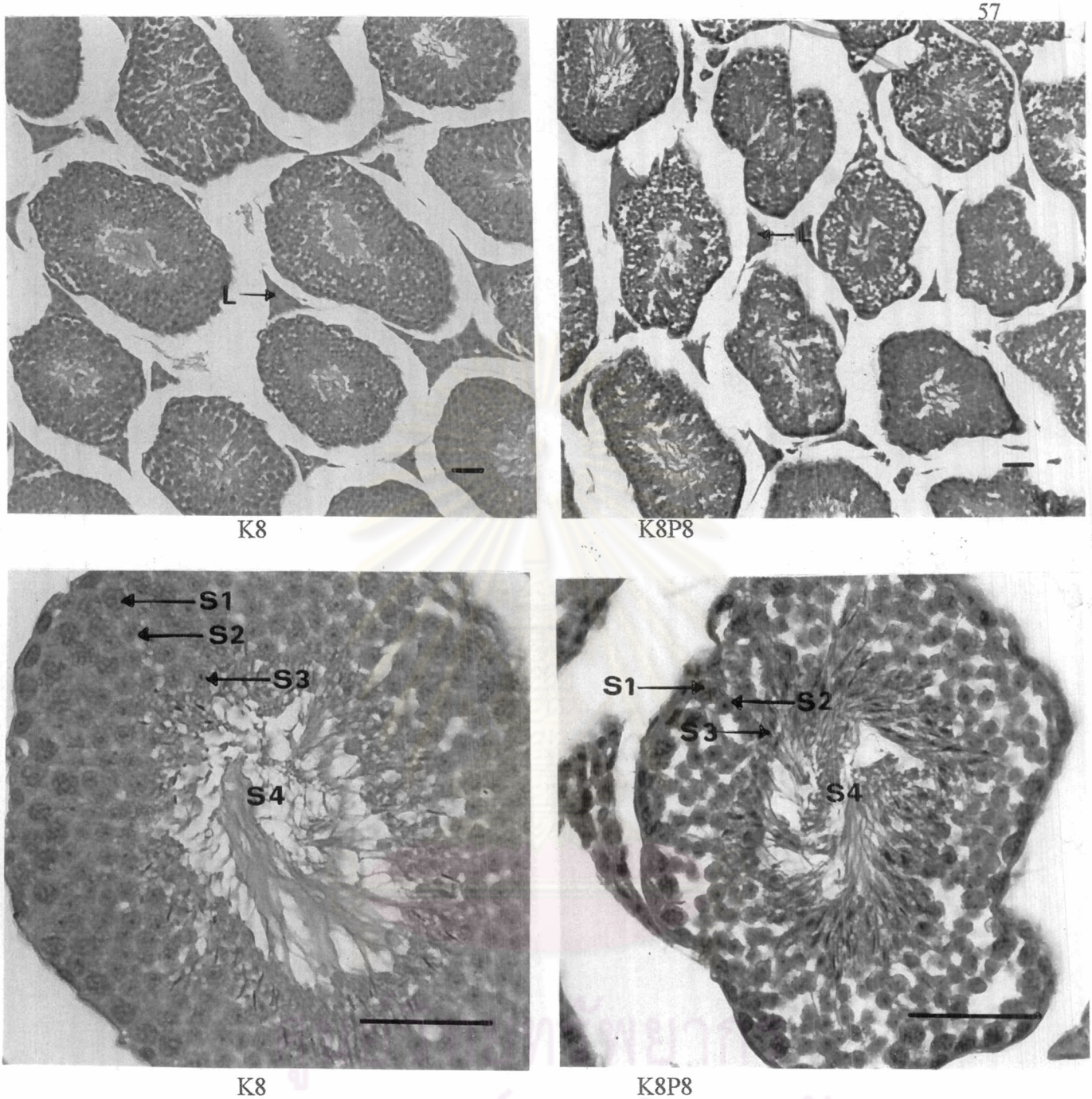


Figure 12. Testicular morphology in mice treated with 100 mg/kg BW/day of *P. mirifica*.

L= Leydig's cells, S1= primary spermatocyte, S2= secondary spermatocyte, S3= spermatid, S4= spermatozoan. H & E stain. (Scale bars = 50 μm)

K8 and K8P8 indicate that male mice were autopsied at the end of treatment and post-treatment periods, respectively.

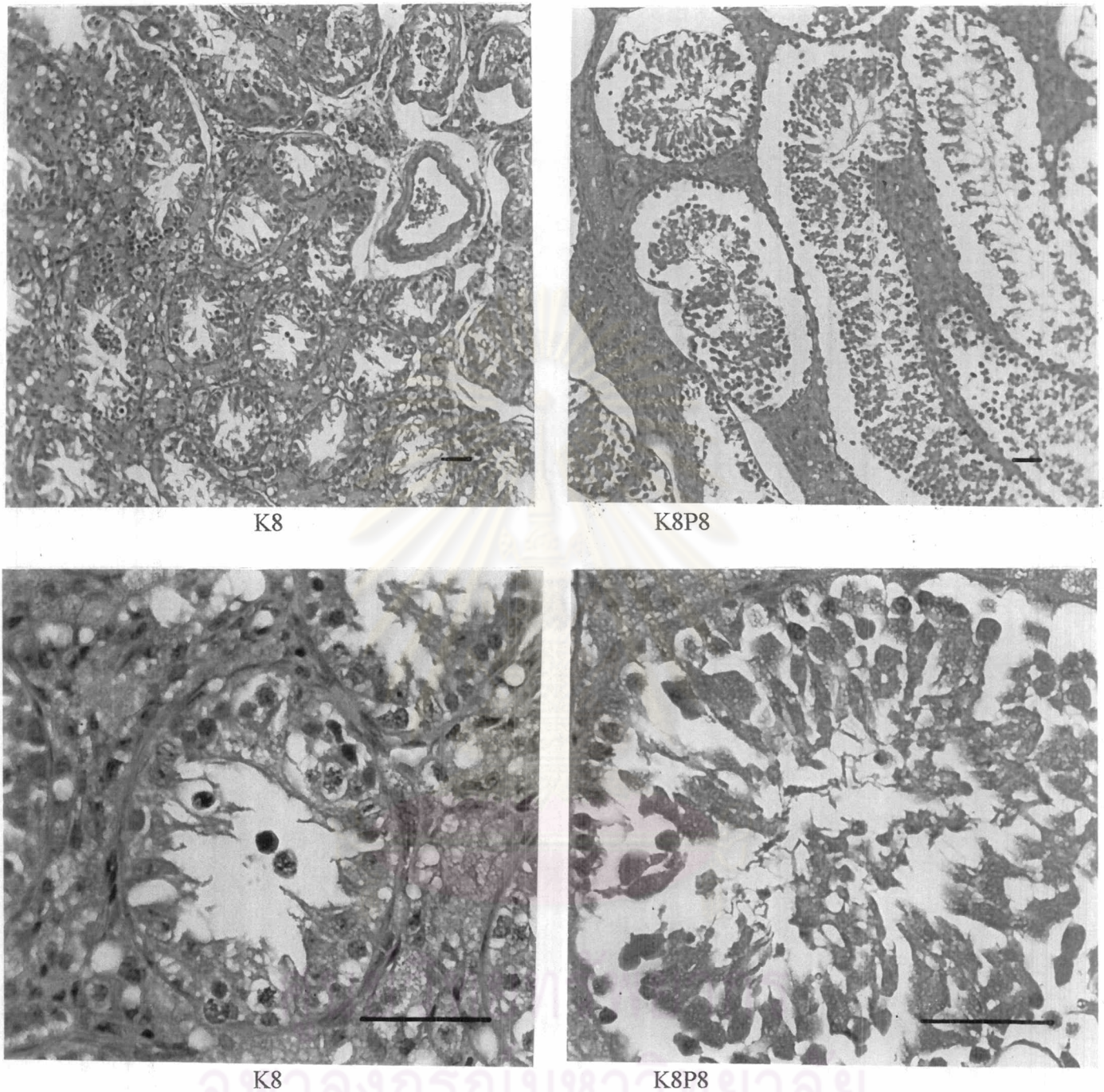


Figure 13. Testicular morphology in mice treated with diethylstilbestrol.

L= Leydig's cells, S1= primary spermatocyte, S2= secondary spermatocyte, S3= spermatid, S4= spermatozoan. H & E stain. (Scale bars = 50 μm)

K8 and K8P8 indicate that male mice were autopsied at the end of treatment and post-treatment periods, respectively.

The number of sperm plug, pregnancy and litter of untreated-female mice mated with treated-male mouse

The number of sperm plug and pregnancy in untreated-female mice mated with male mice treated with 10- and 100-PM seemed to be similar to the DW group at K4 and K8 **(Table7)**. Surprisingly, the number of sperm plug and pregnancy were increased after male mice were withdrawn from 100-PM treatment for 4 wks (K8P4). All of untreated-female mice mated with DES injected male mice showed no sperm plug and pregnancy throughout the treatment period (K4 and K8). After 4 wks of DES withdrawal (K8P4), the male mice could mate with virgin female and showed the sperm plug as same as the DW group (53.33%), but no pregnancy occurred. The pregnancy (40%) could be found only after 8 wks of DES withdrawal (K8P8).

The average number of litters that born from father treated with 10- and 100-PM were similar to that of DW group **(Table8)**. All of DES treated male mice did not produce a litter throughout treatment and the first 4 wks of post-treatment period (K8P4). They could completely recover from the treatment and produce the litter at 8 wks of DES withdrawal (K8P8).

Group	K4 (n=30)		K8 (n=30)		K8P4 (n=15)**		K8P8 (n=15)**	
	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy
DW	16 53.33%*	13 43.33%	18 60%	14 46.67%	8 53.33%	7 46.67%	8 53.33%	8 53.33%
10-PM	17 56.67%	15 50%	16 53.33%	15 50%	8 53.33%	8 53.33%	7 46.67%	7 46.67%
100-PM	13 43.33%	11 36.67%	14 46.67%	14 46.67%	12 80%	12 80%	10 66.67%	10 66.67%
DES	0 0%	0 0%	0 0%	0 0%	8 53.33%	0 0%	11 73.33%	6 40%

Table 7. Sperm plug and pregnancy found in normal female mice after mating with male mouse treated with distilled water, *P. mirifica* and diethylstilbestrol.

* The percent of sperm plug and pregnancy were calculated as the number of females found sperm plug and pregnancy per the number of total used females x 100.

**Half of male mice (n=5) were autopsied at the end of treatment period, and only half (n=5) were remained and mated with virgin female mice in this period (sex ratio = 1:3).

K4 and K8 = Treatment with *P. mirifica* or DES for 4 and 8 weeks, respectively.

K8P4 and K8P8 = Treatment with *P. mirifica* or DES for 8 weeks, and withdrawal for 4 and 8 weeks, respectively.

Group	K4	K8	K8P4	K8P8
DW	13	11	10	10
10-PM	12	11	11	9
100-PM	11	11	13	12
DES	0	0	0	12

Table 8. Average number of litter born form the untreated-female mice mated with treated-male mouse. The male mouse was treated with distilled water, *P. mirifica* and diethylstilbestrol. Explanations for K4, K8, K8P4 and K8P8 are in **Table 7**.

Effects of *P. mirifica* on hormone-related ovarian functions, reproductive organs and fertility in female mice.

Serum LH levels

Compared to the pre-treatment levels (0 wks), serum LH levels in mice treated with DW and 10-PM did not change throughout the study period **(Figure 14)**. In contrast, serum LH levels in mice treated with 100-PM were reduced at 8 wks and during 4-8 wks of DES treatment. However, serum LH levels could recover within 4 wks after the *P. mirifica* and DES withdrawal. Serum LH levels were significantly increased and become higher than the pre-treatment level at 8 wks of DES withdrawal.

Compared to the DW group, serum LH levels in mice treated with 10-PM were significantly higher at 8 wks of treatment and at 8 wks of post-treatment period. Serum LH levels in mice treated with 100-PM were significantly lower than the DW group at 8 wks of treatment period and recovered after the *P. mirifica* withdrawal. In contrast, serum LH levels in mice treated with DES were significantly lower than the DW group at 4-8 wks of treatment period, and the mice showed a non-significant difference of LH levels at 4-8 wks of post-treatment period.

Compared to the DES group, serum LH levels in mice treated with 10-PM were significantly higher throughout treatment period (4-8 wks), and no differences during the post-treatment period. However, the pattern of serum LH levels in mice treated with 100-PM were not differences from the DES group throughout the study period.

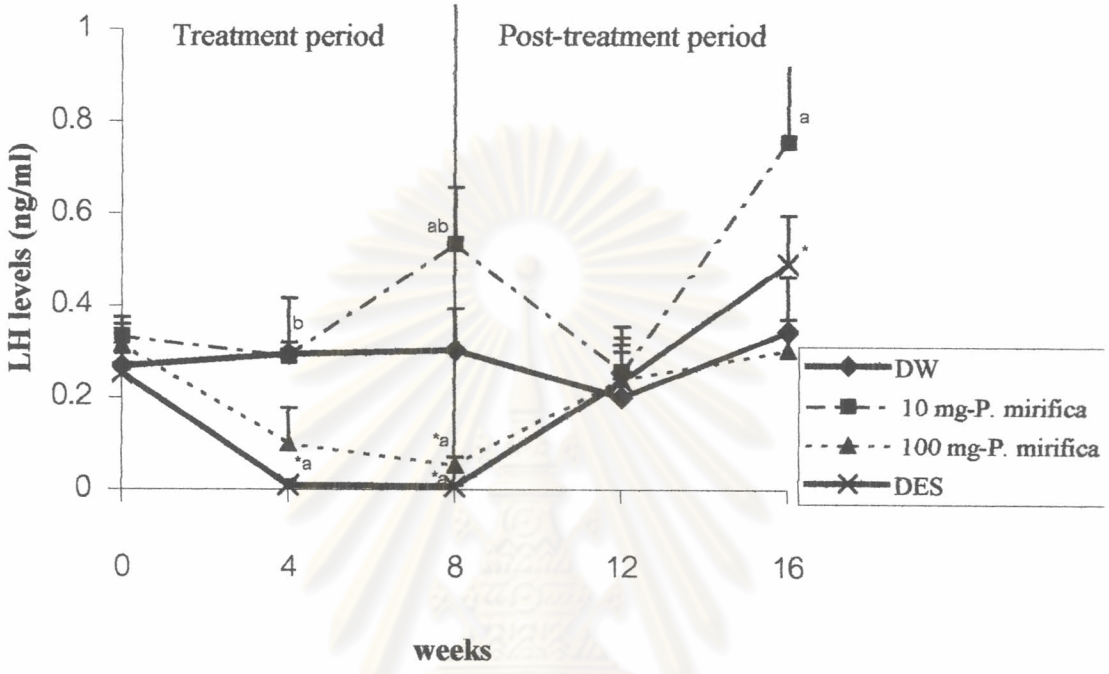


Figure 14. Serum LH concentration of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Serum FSH levels

Compared to the pre-treatment levels (0 wks), serum FSH levels in DW group were significantly increased at 8 wks of treatment and at 8 wks of post-treatment period **(Figure 15)**. Serum FSH in mice treated with 10- and 100-PM and DES did not change throughout the treatment period, however, the levels in post-treatment period were significantly increased at 8 wks in mice treated with 10-PM and at 4 wks in mice treated with 100-PM and DES.

Compared to the DW group, serum FSH levels in mice treated with 10-PM, 100-PM and DES were significantly lower at 8 wks of treatment period.

Compared to the DES group, serum FSH levels in mice treated with 10- and 100-PM showed no difference throughout the study period.

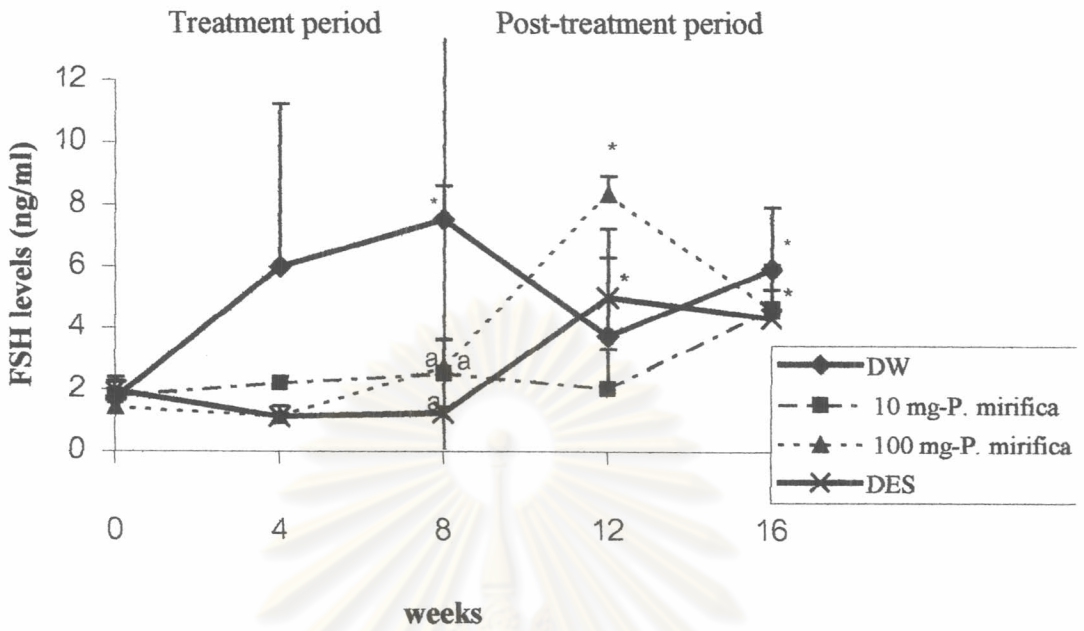


Figure 15. Serum FSH concentration of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Serum E_2 levels

Since the levels of E_2 at 0 wks among those 4 groups were significantly different the E_2 levels in each group at 0 wks were adjusted to zero and the other data were calculated and transformed as a percent change from levels of 0 wks.

Compared to the pre-treatment levels (0 wks), serum E_2 levels in mice treated with DW, 10-PM and 100-PM did not change throughout the study period **(Figure 16)**. In contrast, serum E_2 levels in mice treated DES were significantly increased at 4 wks of treatment period, and did not change during the post-treatment period.

Compared to the DW group, serum E_2 levels in mice treated with 10- and 100-PM did not difference throughout the study period. In contrast, serum E_2 levels in mice treated with DES were significantly higher at 4 wks of treatment period, and did not difference during the post-treatment period.

Compared to the DES group, serum E_2 levels in mice treated with 10- and 100-PM were significantly lower at 4 wks of treatment period, and did not difference during the post-treatment period.

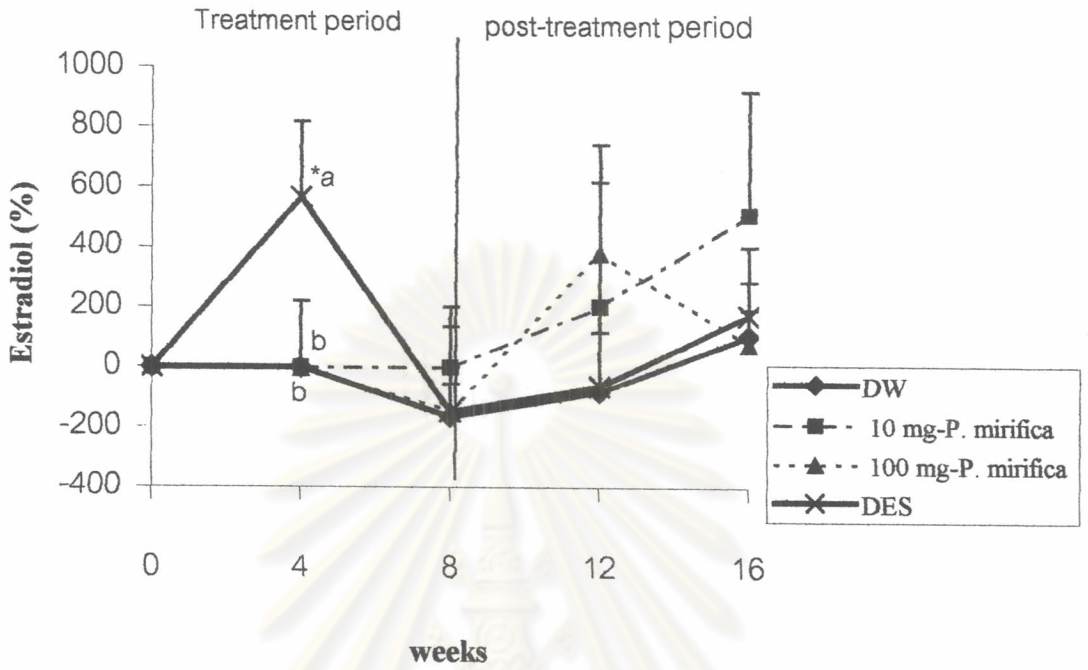


Figure 16. Percent change of serum E_2 concentration of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from 0 wks ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Body weights and organ weights

Compared to day-1, body weights in mice treated with DW, 10- and 100-PM did not change throughout the study period **(Figure 17)**. In contrast, body weights in DES injected mice were significantly increased at day-8 and reached the plateau at day-15.

Compared to the DW group, body weights in mice treated with 10- and 100-PM did not difference throughout the study period. However, body weights in DES injected mice were totally significantly higher than the DW and PM groups since day-8 of the study period.

Compared the weights of uterus and ovary between the end of treatment period (K8A) and the end of post-treatment period (K8P8A), it was found that the uterus and ovary weights in mice treated with DW, 10- and 100-PM and DES did not difference, except the ovary weights in 10-PM were increased **(Table 9)**.

Compared to the DW group, weights of uterus and ovary in all *P. mirifica* treated and DES injected mice were not significant differences from the DW group.

Compared to the DES group, only the weights of uterus and ovary collected at the end of treatment period (K8A) in mice treated with 10-PM were significantly lower than the DES group.

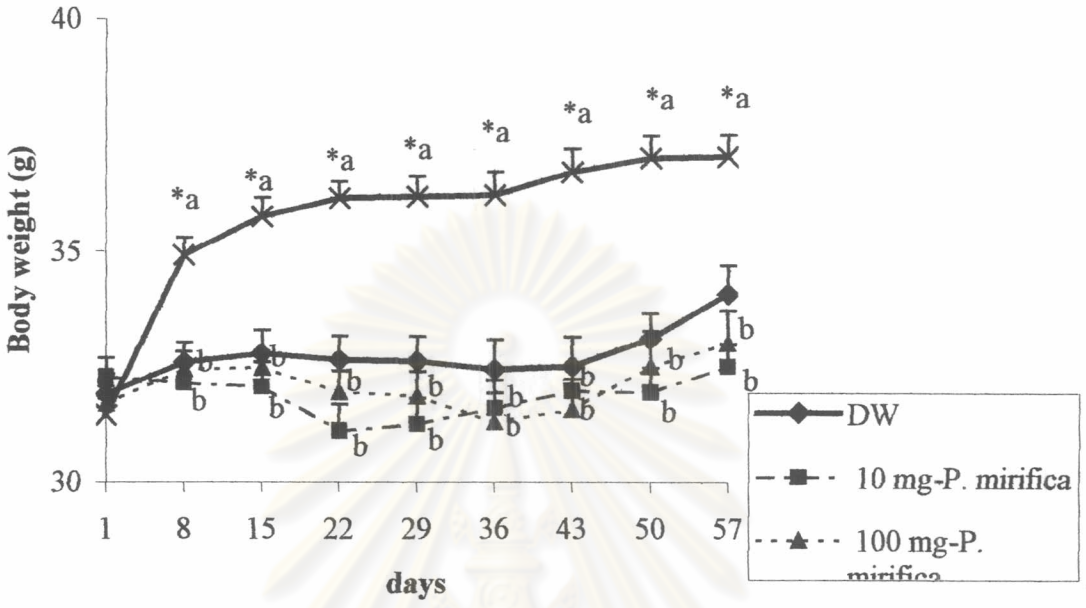


Figure 17. Body weights of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from day-1 ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Group	Uterus (g)		Ovary (g)	
	K8A	K8P8A	K8A	K8P8A
DW	0.216±0.029	0.179±0.037	0.026±0.001	0.026±0.003
10-PM	0.158±0.022[b]	0.180±0.027	0.022±0.002[b]	0.031±0.002[*]
100-PM	0.193±0.030	0.160±0.034	0.027±0.003	0.024±0.002
DES	0.246±0.028	0.204±0.047	0.034±0.006	0.025±0.003

Table 9. Weights of uterus and ovary of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol. K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

[*] Significantly different from K8A ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

Compared the relative organ weights between K8A and K8P8A, it was found that the relative weights of uterus and ovary in mice treated with DW, 10- PM, 100-PM and DES did not different, except in 10-PM the relative weight of ovary was increased **(Table 10)**.

Compared to the DW group, the relative weights of uterus and ovary in all *P. mirifica* treated and DES injected groups were not significant differences.

Compared to the DES group, the relative weights of uterus and ovary in all *P. mirifica* treated groups were not significant differences.

Vaginal smear

The DW and 10-PM treatment did not affect on the vaginal epithelium, mice show a regular estrous cycle (4-5 days) throughout study period **(Figure 18-19)**. In contrast, 100-PM and DES treatment induced a cornification of the vaginal smear, mice showed an unestrous cycle, since day-2 of treatment period **(Figure 20-21)**. Mice could recover from treatment and become to have a regular estrous cycle after 3-4 days of 100-PM withdrawal. They could recover from treatment and the vaginal cornification was disappeared at 5-8 days of the DES withdrawal, however, the prolonged estrous cycles or persistent appearance of leukocyte cells were shown afterward.

Group	Uterus ($x10^{-2}$)		Ovary ($x10^{-2}$)	
	K8A	K8P8A	K8A	K8P8A
DW	0.615±0.084	0.511±0.094	0.073±0.002	0.074±0.006
10-PM	0.519±0.061	0.544±0.077	0.075±0.007	0.094±0.006*
100-PM	0.589±0.084	0.479±0.107	0.084±0.006	0.074±0.008
DES	0.649±0.069	0.554±0.130	0.090±0.014	0.066±0.007

Table 10. Relative weights of uterus and ovaries of female mice treated with distilled water, *P. mirifica* and diethylstilbestrol. K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

* Significantly different from K8A ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)

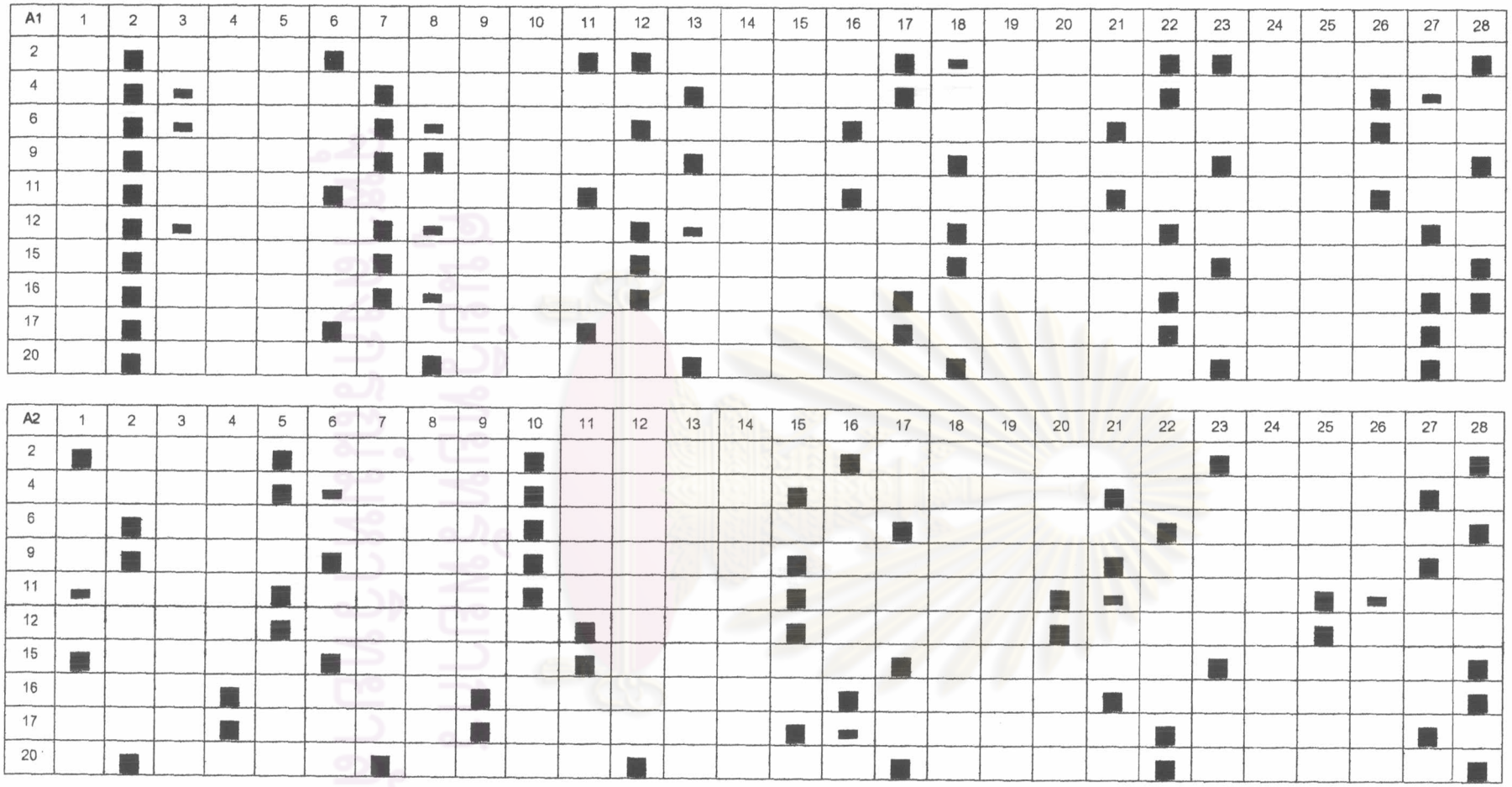


Figure 18. Daily monitoring of vaginal cytology from female mice treated with distilled water during treatment period (A1) and post-treatment period (A2). Four-week data of each period are shown. Numbers at the left corner represent individual animals. Full bars indicate fully cornification, and half bars indicate partial cornification.

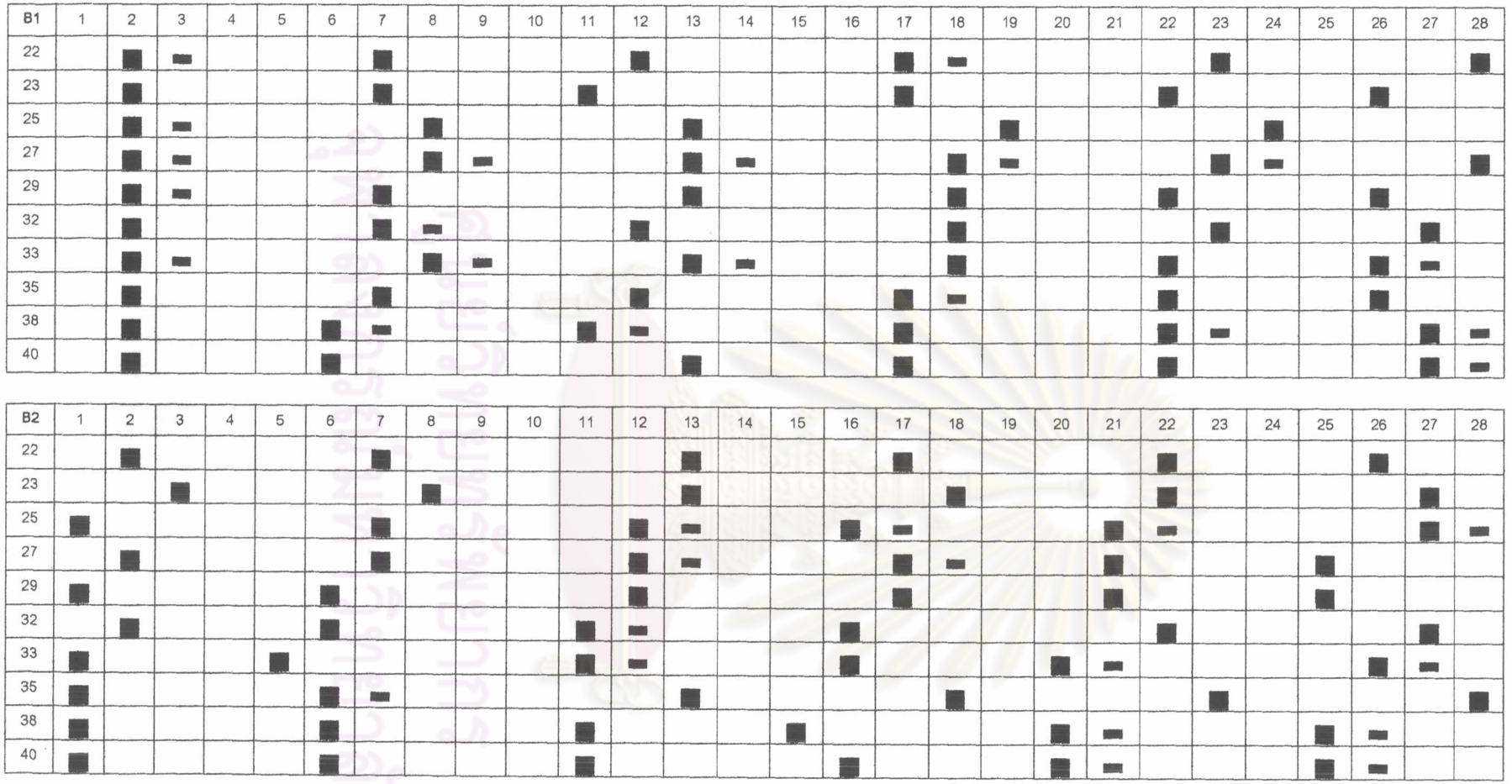


Figure 19. Daily monitoring of vaginal cytology from female mice treated with 10 mg/kg BW/day of *P. mirifica* during treatment period (B1) and post-treatment period (B2). Four-week data of each period are shown. Numbers at the left corner represent individual animals. Full bars indicate fully cornification, and half bars indicate partial cornification.

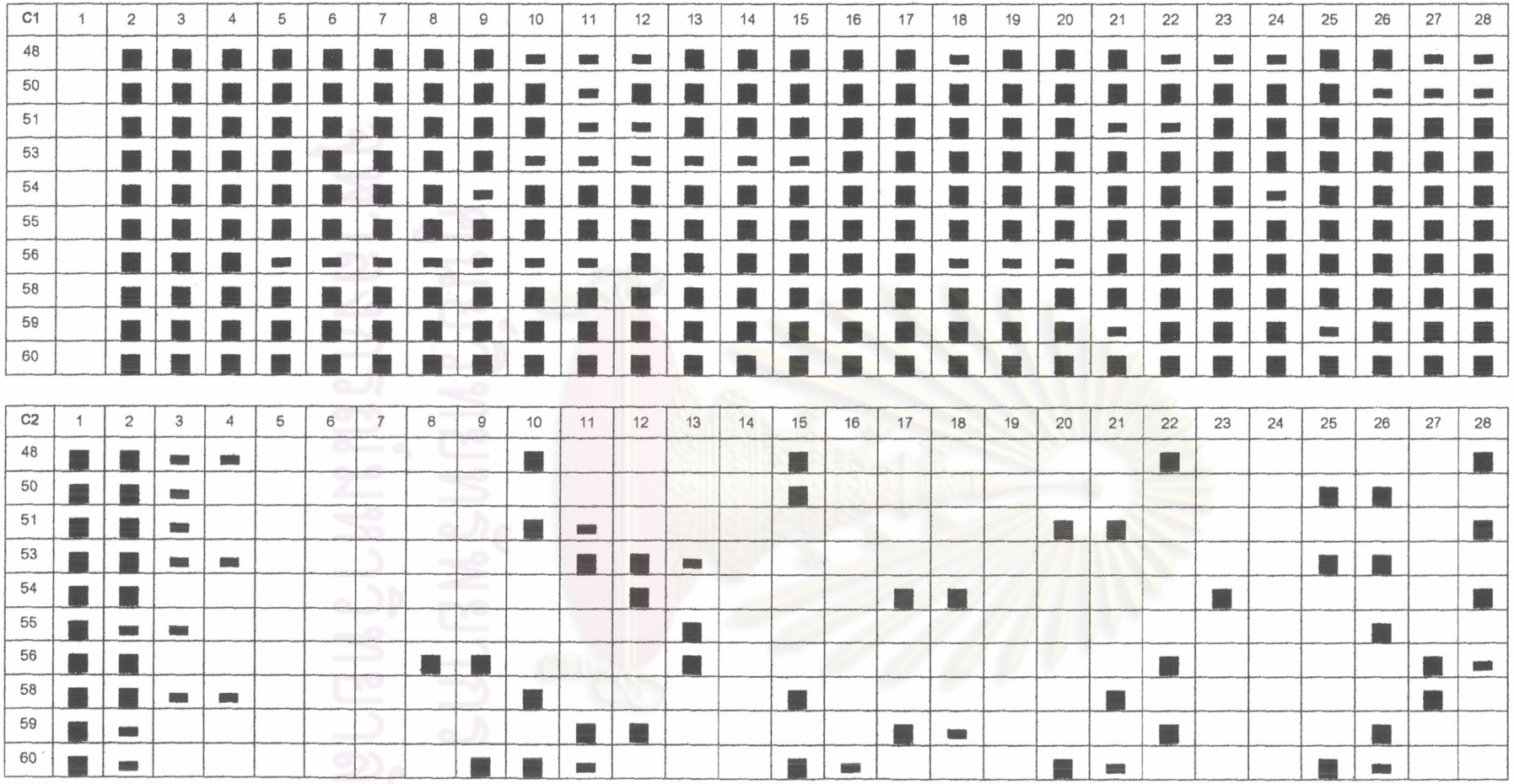


Figure 20. Daily monitoring of vaginal cytology from female mice treated with 100 mg/kg BW/day of *P. mirifica* during treatment period (C1) and post-treatment period (C2). Four-week data of each period are shown. Numbers at the left corner represent individual animals. Full bars indicate fully cornification, and half bars indicate partial cornification.

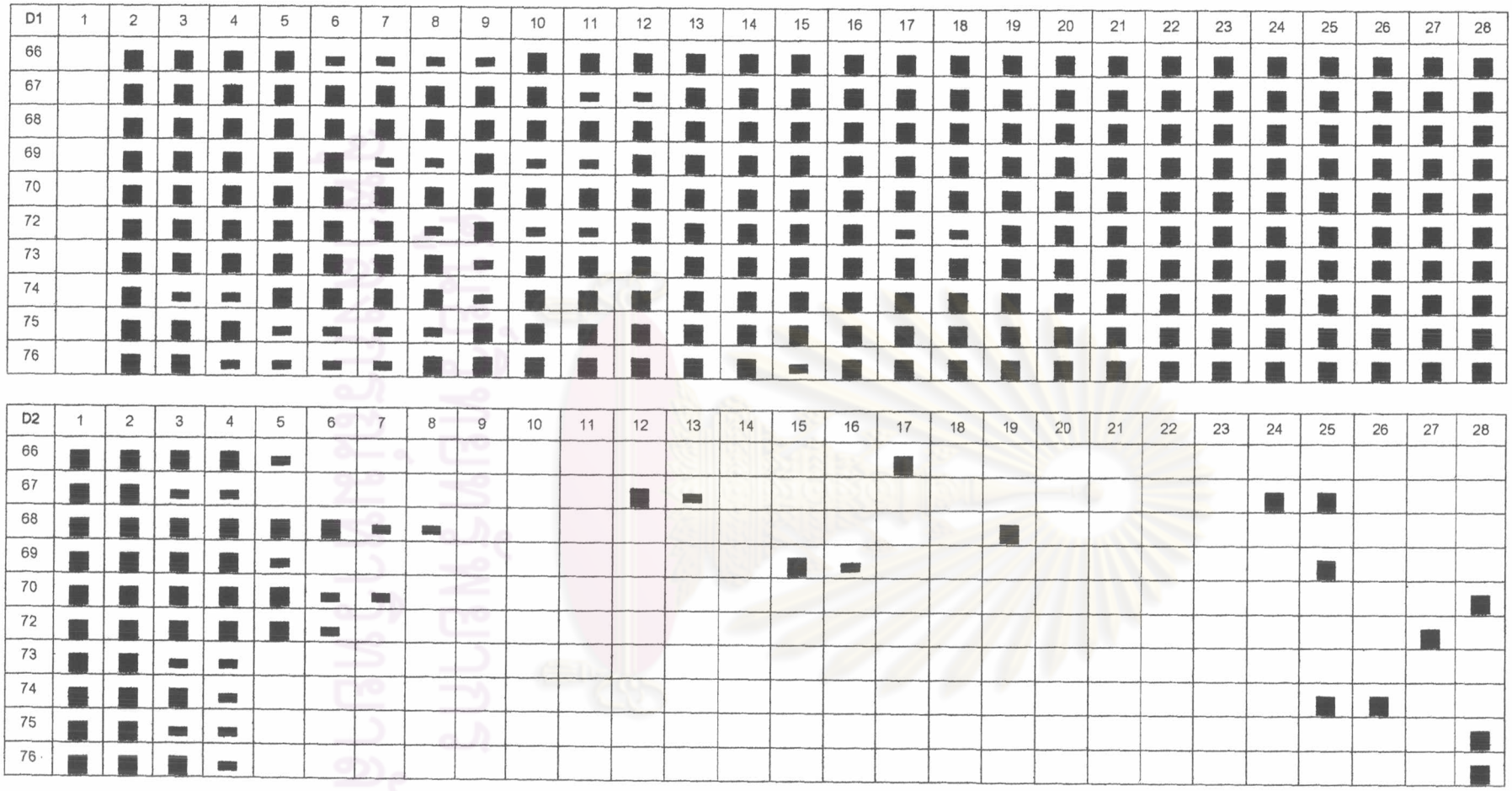


Figure 21. Daily monitoring of vaginal cytology from female mice treated with diethylstilbestrol during treatment period (D1) and post-treatment period (D2). Four-week data of each period are shown. Numbers at the left corner represent individual animals. Full bars indicate fully cornification, and half bars indicate partial cornification.

Characteristics of uterine proliferation and folliculogenesis

The histological study of uterus in DW and 10-PM groups at K8 showed the same lines with a simple columnar epithelium overlaying the thick lamina propria of the endometrium. The uterine glands were wavy in outline and winden in their lamina propia **(Figure 22-23)**. In contrast, uterus of mice treated with 100-PM and DES at K8 showed thicker of endometrium and dilated of uterine glands than control group **(Figure 24-25)**. In addition, the uterine glands in mice treated with DES showed a lot of secretory material accumulation **(Figure 25)**. However, these occurrences could be recovered within 8 wks (K8P8) after *P. mirifica* and DES withdrawal.

The ovarian morphology of mice in DW and 10-PM groups at K8 weeks showed numerous of ovarian follicles in various stages such as primordial follicles, primary follicles, secondary follicles, Graafian follicles and copus luteum **(Figure 26-27)**. The decreasing of primary, secondary and Graafian follicles are seen in ovary of mice treated with 100-PM at K8 **(Figure 28)**. In contrast, the ovary in mice treated with DES showed atretic follicles and atrophic morphologic changes in granulosa cells **(Figure 29)**. However, all these morphological changing of ovary could be recovered within 8 wks (K8P8) after *P. mirifica* and DES withdrawal.

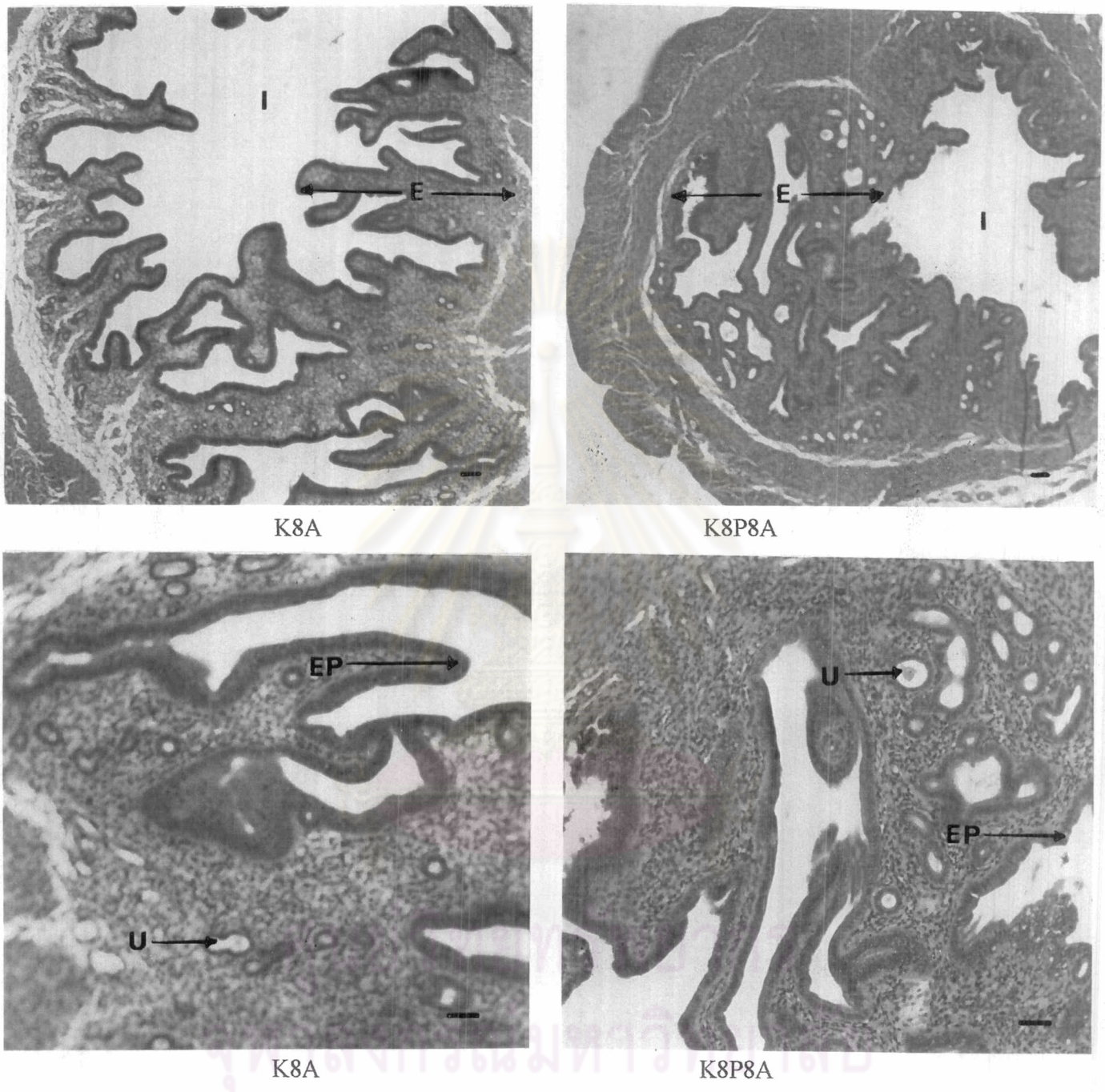


Figure 22. Uterus morphology in DW group. EP = epithelial cell, E = endometrium, I = uterine lumen, U = uterine gland. H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

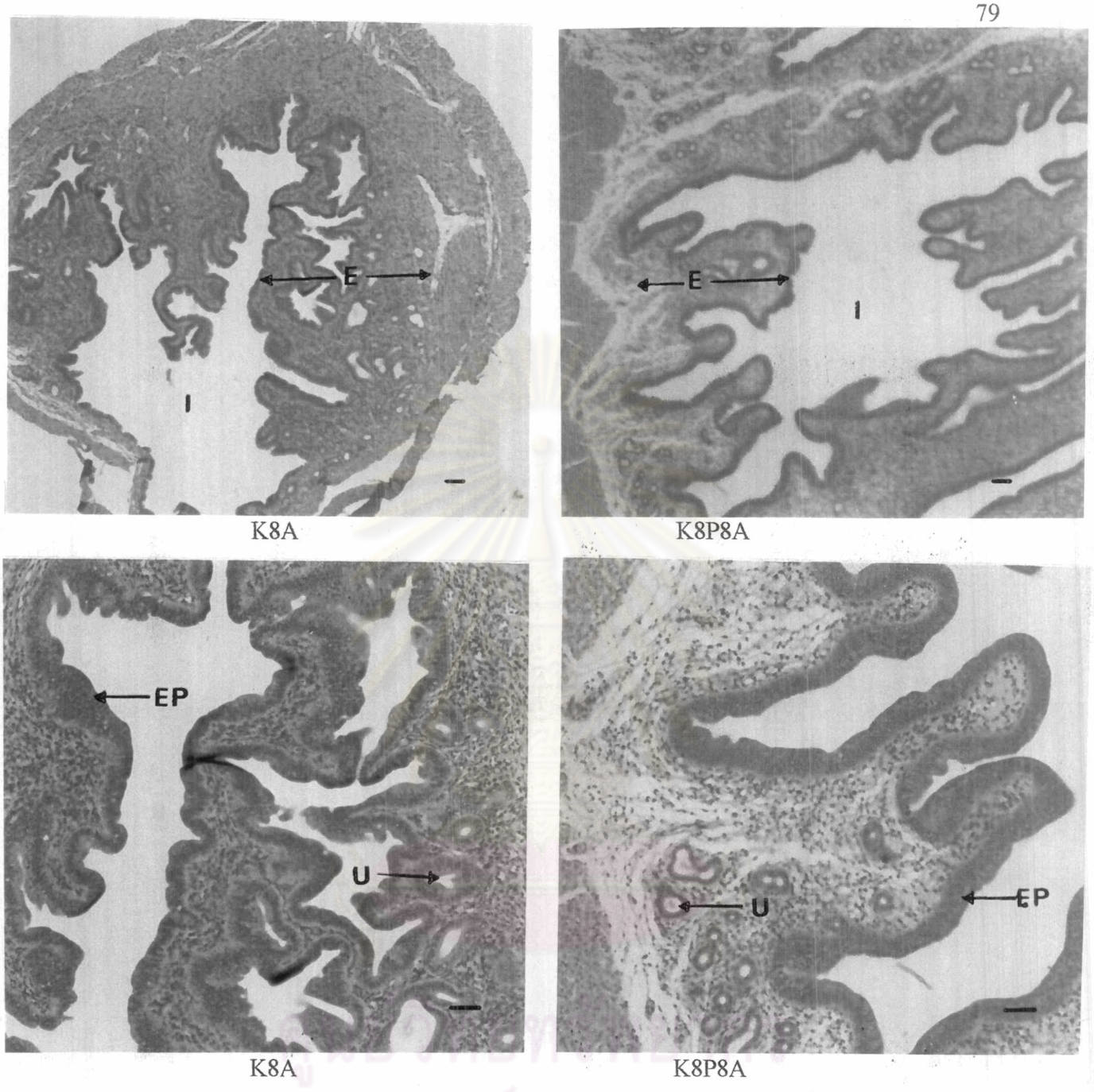


Figure 23. Uterus morphology in mice treated with 10 mg/kg BW/day of *P. mirifica*.

EP = epithelial cell, E = endometrium, I = uterine lumen, U = uterine gland

H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

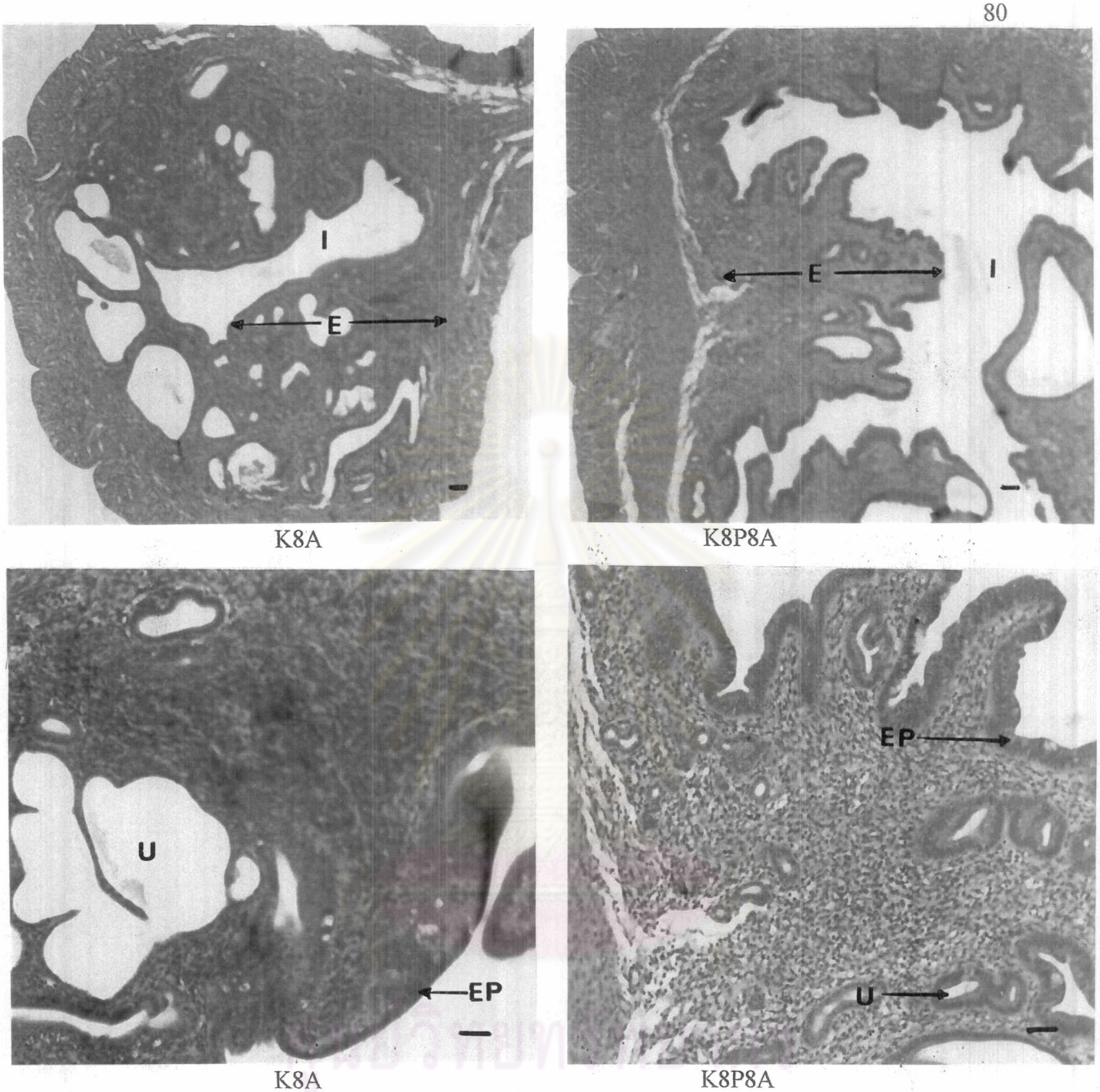
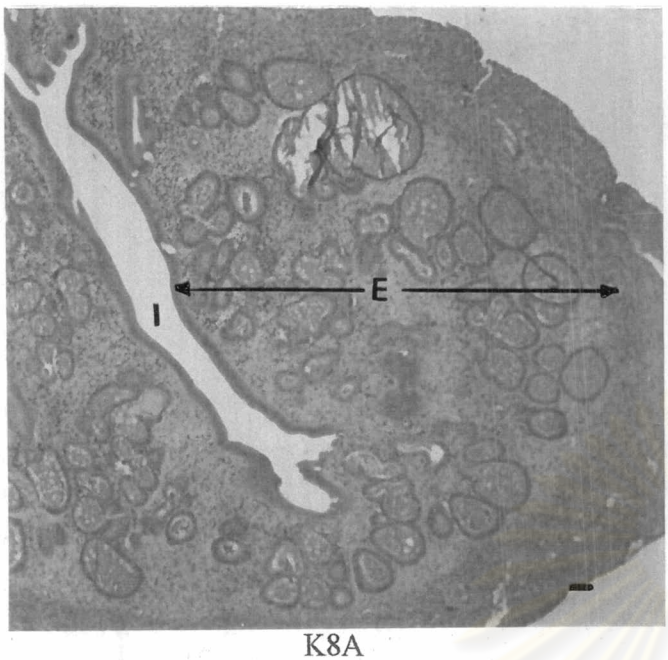


Figure 24. Uterus morphology in mice treated with 100 mg/kg BW/day of *P. mirifica*.

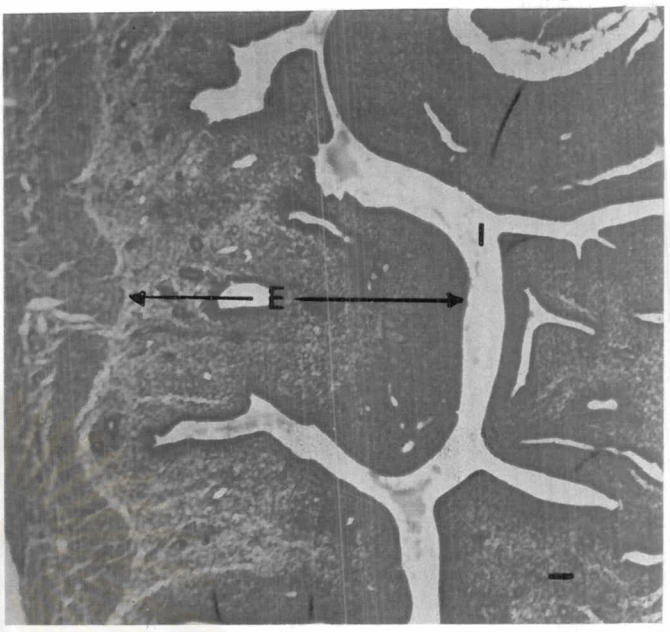
EP = epithelial cell, E = endometrium, I = uterine lumen, U = uterine gland.

H & E stain. (Scale bars = 50 μm)

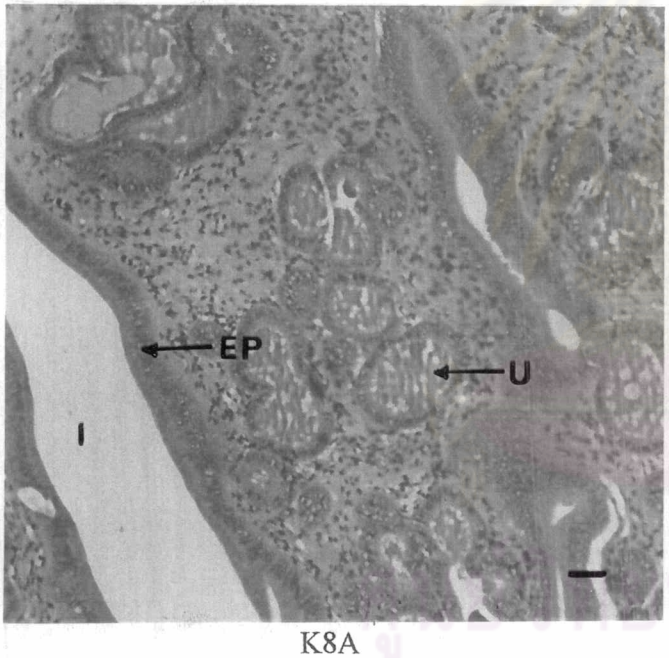
K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.



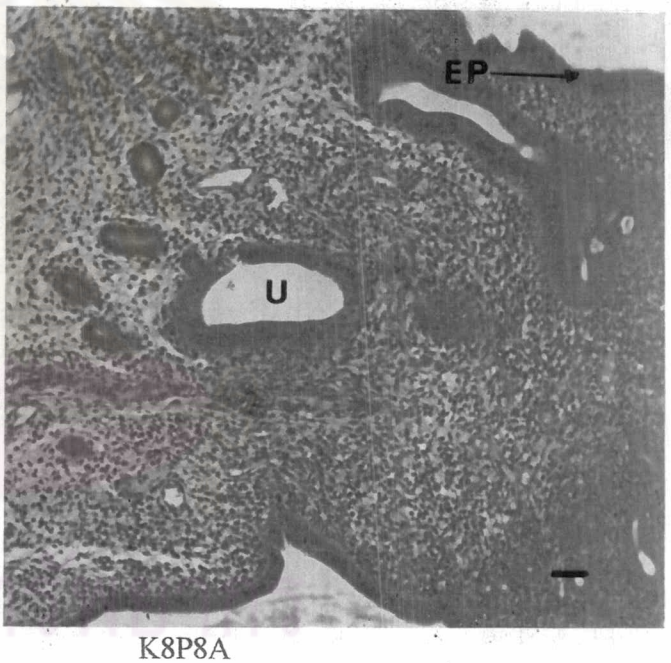
K8A



K8P8A



K8A



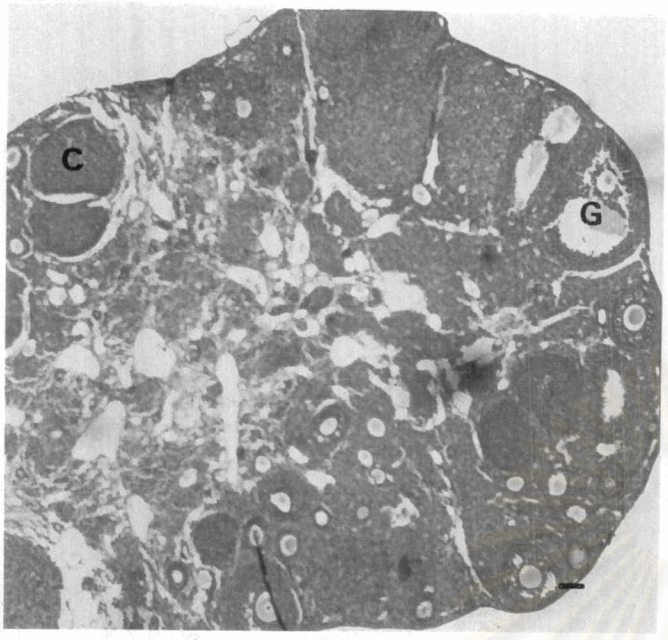
K8P8A

Figure 25. Uterus morphology in mice treated with diethylstilbestrol.

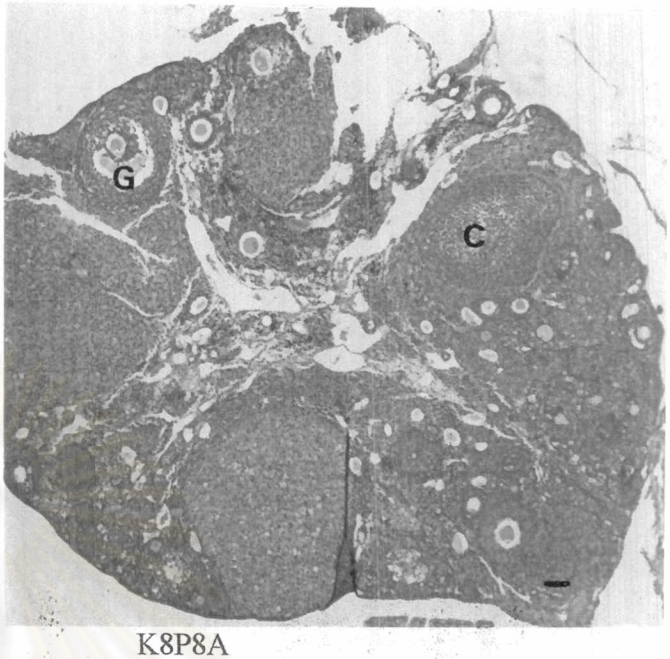
EP = epithelial cell, E = endometrium, I = uterine lumen, U = uterine gland.

H & E stain. (Scale bars = 50 μm)

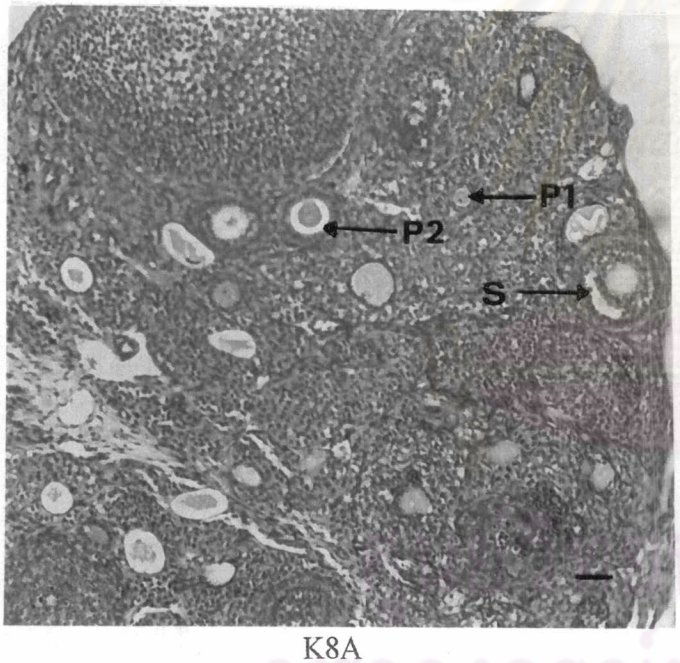
K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.



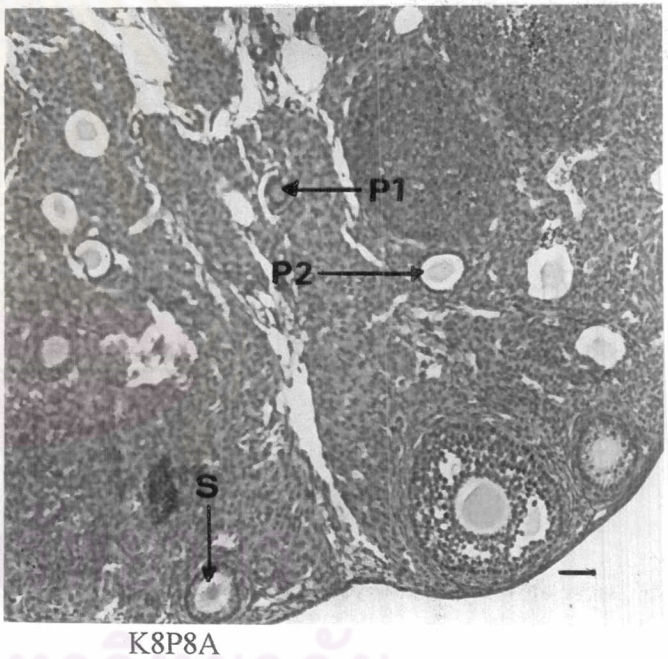
K8A



K8P8A



K8A



K8P8A

Figure 26. Ovarian morphology in DW group. P1 = primodial follicle, P2 = primary follicle, S = secondary follicle, G =graafian follicle, C= copus luteum. H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

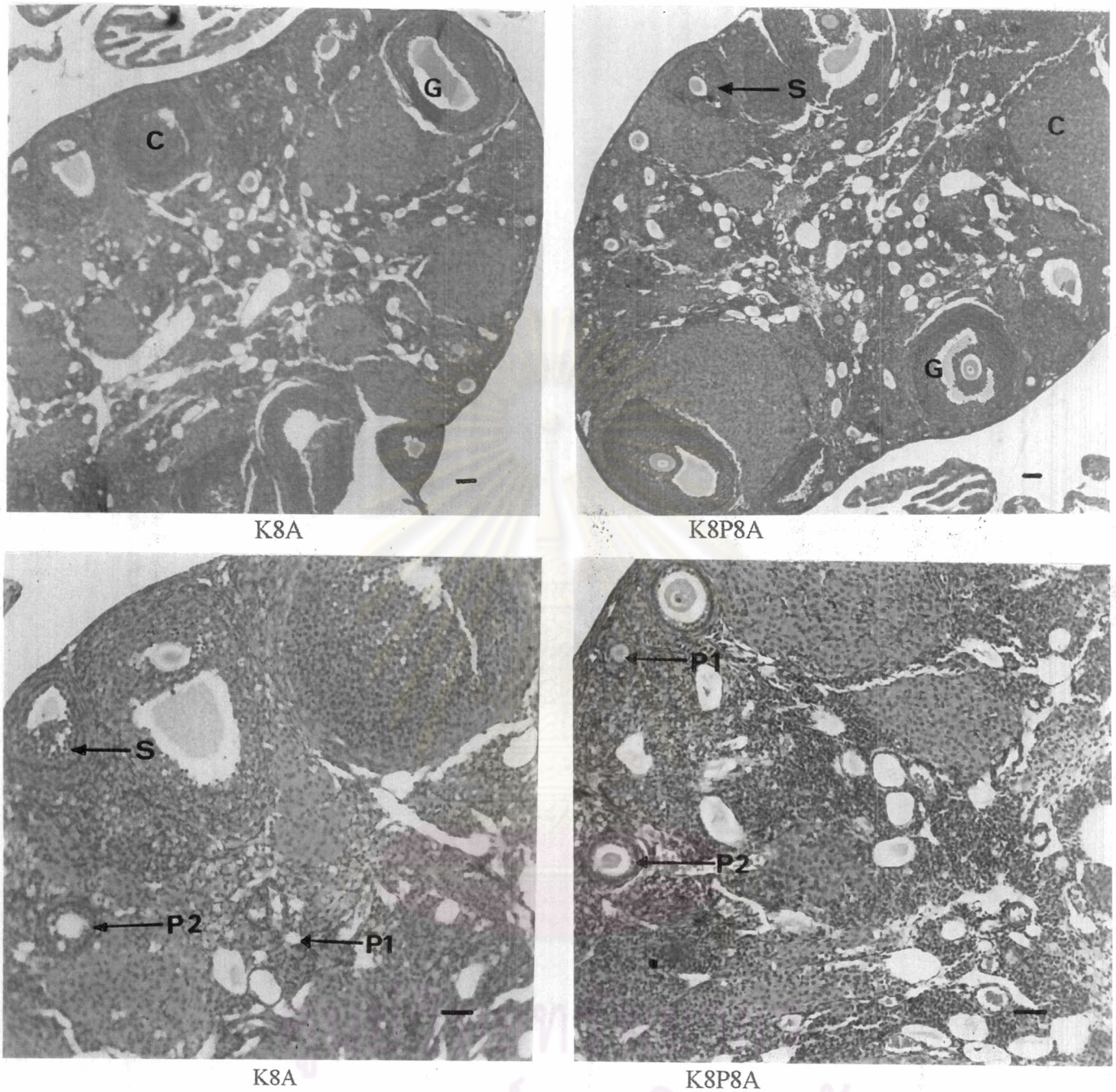
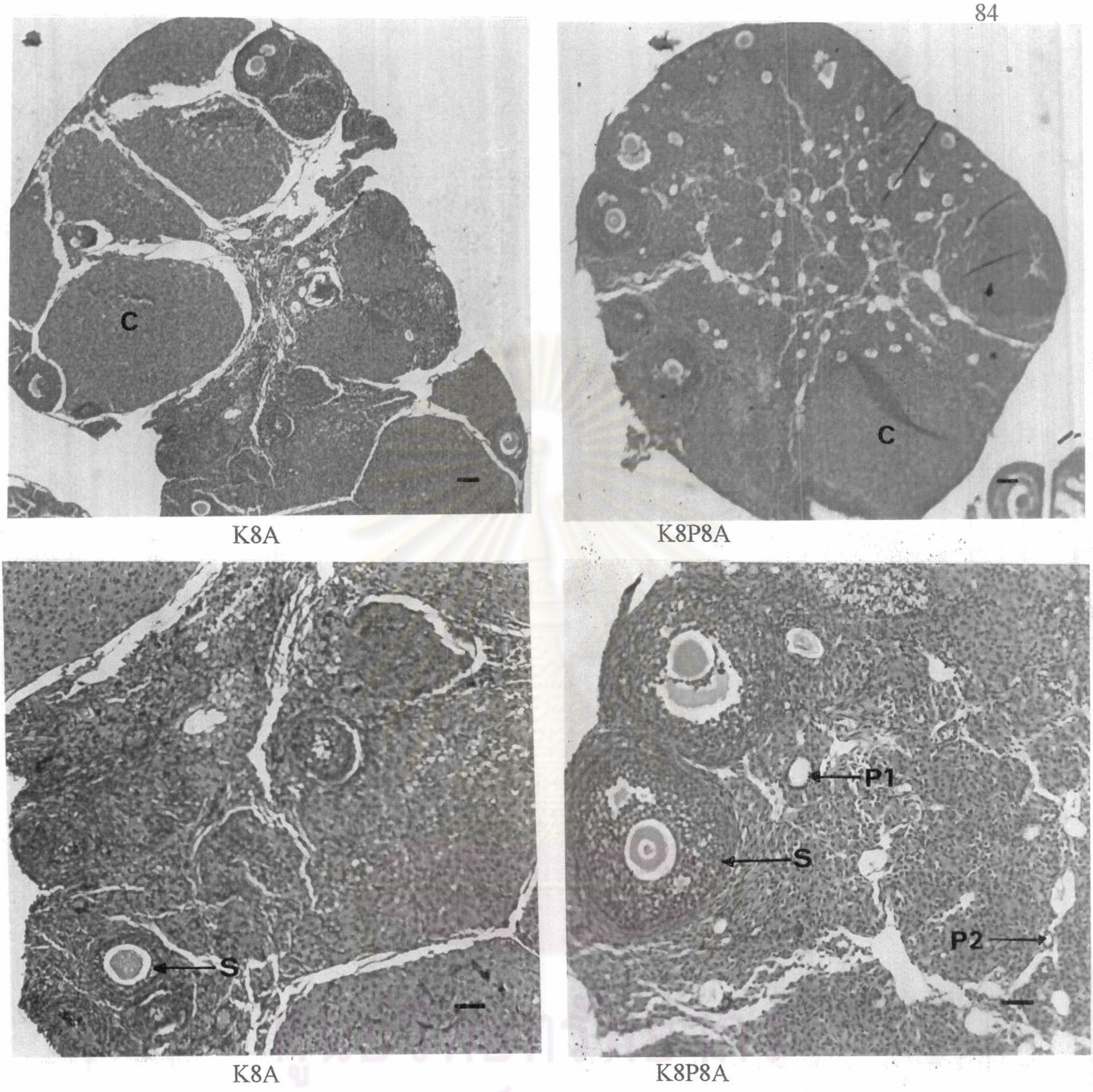


Figure 27. Ovarian morphology in mice treated with 10 mg/kg BW/day of *P. mirifica*.

P1 = primodial follicle, P2 = primary follicle, S = secondary follicle, G =graafian follicle,

C= copus luteum. H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.



K8A K8P8A

K8A K8P8A

Figure 28. Ovarian morphology in mice treated with 100 mg/kg BW/day of *P. mirifica*.

P1 = primodial follicle, P2 = primary follicle, S = secondary follicle, G =graafian follicle,

C= copus luteum. H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

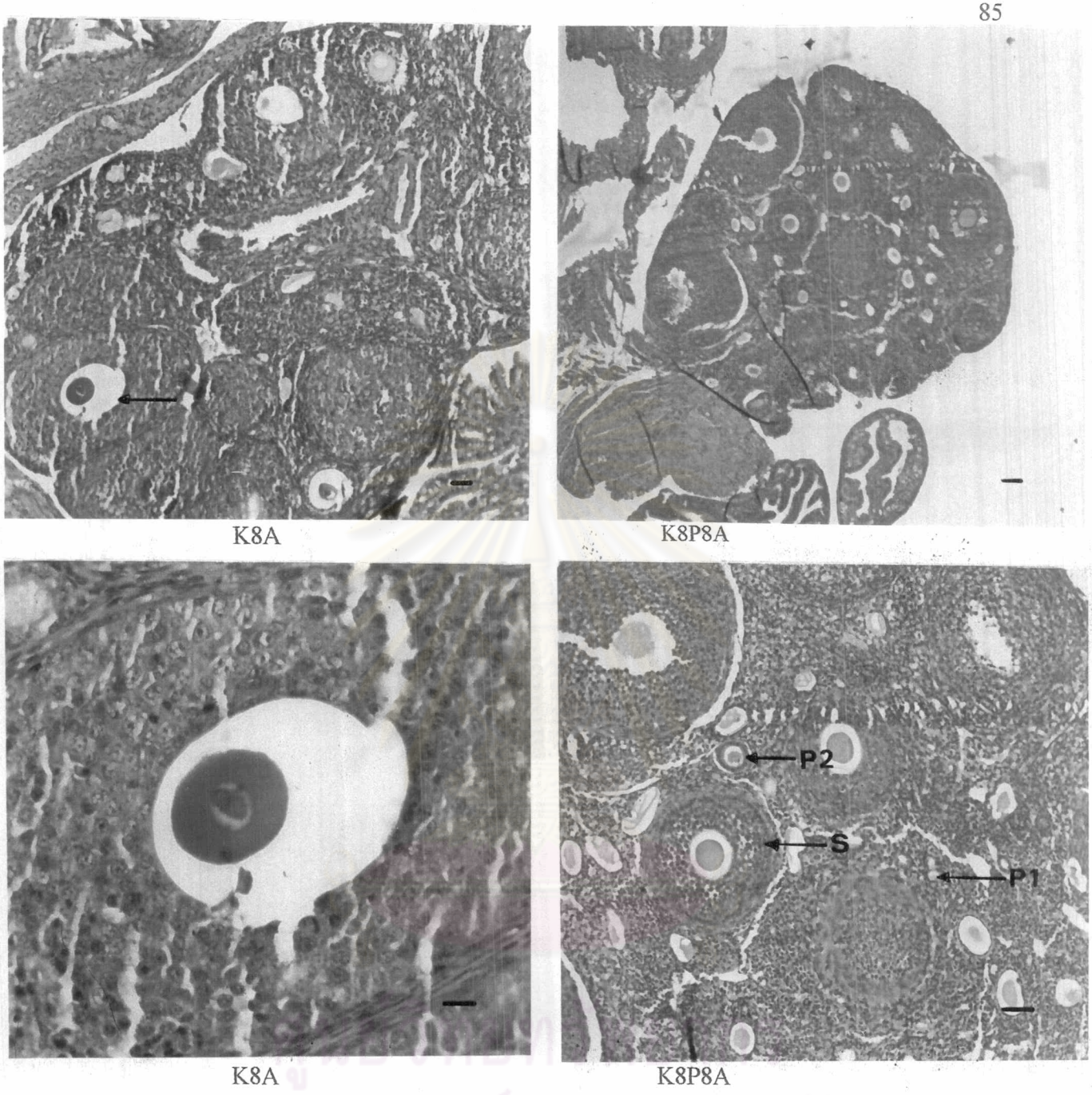


Figure 29. Ovarian morphology in mice treated with diethylstilbestrol.

P1 = primodial follicle, P2 = primary follicle, S = secondary follicle, G =graafian follicle,

C = copus luteum, atretic follicle (arrow). H & E stain. (Scale bars = 50 μm)

K8A and K8P8A indicate that female mice were autopsied at the end of treatment and post-treatment periods, respectively.

The number of sperm plug, pregnancy and litter born of treated-female mice mated with untreated-male mouse

The number of sperm plug and pregnancy in female mice treated with 10-PM for 8 wks could increase when compared to the DW group for 33.33 and 66.66%, respectively **(Table 11)**. In contrast, treatment of 100-PM can reduce the sperm plug for 50% and pregnancy rate for 33.34% within 4 wks, and no sperm plug and pregnancy were found after 8 wks of treatment. However, those parameters could be recovered within 4 wks after withdrawal of *P. mirifica* administration. Some female mice treated with DES for 4 and 8 wks could accept the mating from untreated-male mouse, but no pregnancy was occurred. Moreover, the DES treated female mice could not recover and be fertile within 8 wks after the DES withdrawal.

The average number of litter born form the treated-female mice treated with 10-PM was similar to that of DW group **(Table 12)**. In contrast,the average number of litter was reduced at 4 wks and no litter was born at 8 wks of treatment period of 100-PM group. However, the average number of litter was able to recover within 4 wks after *P. mirifica* withdrawal. Female mice treated with DES for 4 and 8 wks had no number of litter. Moreover, they could partially recover from the treatment and produce the litter at 4 and 8 wks of DES withdrawal.

GROUP	K4 (n=6)		K8 (n=6)		K8P4 (n=6)		K8P8 (n=6)	
	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy	Sperm plug	Pregnancy
DW	5 83.33%*	4 66.67%	3 50%	1 16.67%	4 66.67%	3 50%	4 66.67%	5 83.33%
10-PM	4 66.67%	4 66.67%	5 83.33%	5 83.33%	3 50%	3 50%	3 50%	3 50%
100-PM	2 33.33%	2 33.33%	0 0%	0 0%	4 66.67%	3 50%	4 66.67%	4 66.67%
DES	1 16.67%	0 0%	2 33.33%	0 0%	1 16.67%	1 16.67%	1 16.67%	1 16.67%

Table 11. Sperm plug and pregnancy found in female mice treated with distilled water, *P. mirifica* and diethylstilbestrol after mating with fertile male mouse. Explanations for K4, K8, K8P4 and K8P8 are in **Table 7**.

* The percent of sperm plug and pregnancy were calculated as the number of females found sperm plug and pregnancy per the number of total used females x 100.

Group	K4	K8	K8P4	K8P8
DW	11	10	9	8
10-PM	11	8	11	11
100-PM	9	0	9	9
DES	0	0	3	5

Table 12. Average numbers of litter born from the treated-female mice mated with untreated-male mouse. The female mice were treated with distilled water, *P. mirifica* and diethylstilbestrol. Explanations for K4, K8, K8P4 and K8P8 are in **Table7**.

Effects of *P. mirifica* on reproductive organs and malformation of litters born form *P. mirifica*-treated parents.

1. *P. mirifica* or DES-treated father

The relative weights of reproductive organs in both sexes of 50-day pups (ovary and uterus in females, and testis epididymis and seminal vesicles in males) born from *P. mirifica* and DES treated fathers did not difference from the DW group **(Table 13)**.

The body weights of litters in all 4 groups were significantly higher than day-1, and increased the weight throughout the study **(Figure 30)**. However, the increase of body weights of litters born from *P. mirifica* and DES treated fathers did not difference from that of DW group. No malformation of pups was found.

Group	Uterus ($x10^{-2}$)	Ovary ($x10^{-2}$)	Testis ($x10^{-2}$)	Epididymis ($x10^{-2}$)	Seminal vesicle ($x10^{-2}$)
DW	0.581±0.002	0.063±0.000	0.777±0.001	0.231±0.001	0.651±0.001
10-PM	0.582±0.001	0.063±0.000	0.761±0.001	0.269±0.002	0.618±0.001
100-PM	0.577±0.003	0.064±0.000	0.750±0.003	0.287±0.003	0.626±0.002
DES	0.632±0.003	0.062±0.000	0.785±0.003	0.261±0.001	0.619±0.002

Table 13. Relative weights of ovary and uterus in females, and testis, epididymis and seminal vesicles in males of the litters born form fathers treated with distilled water, *P. mirifica* and diethylstilbestrol.

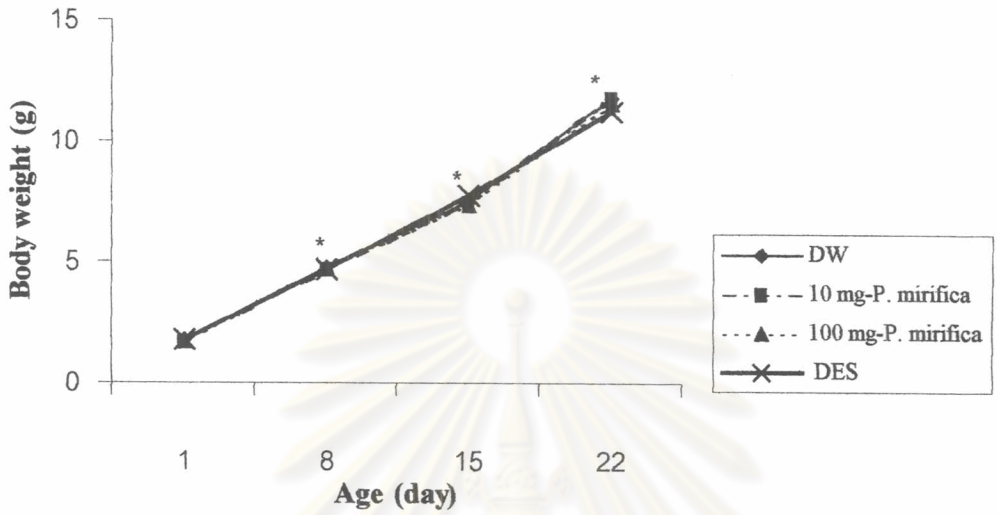


Figure 30. Post-partum growth rate of litters born from fathers treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from day-1 ($p < 0.05$)

2. *P. mirifica* or DES-treated mother

Similar to the results in male mice, the relative weights of reproductive organs in both sexes of 50-day pups (ovary and uterus in females, and testis epididymis and seminal vesicles in males) born from *P. mirifica* and DES treated mothers did not difference from the DW group **(Table14)**.

The body weights of litters in all 4 groups was significantly higher than day-1, and increased the weight throughout the study **(Figure 31)**. However, the increase of the body weights of litters born from *P. mirifica* treated mother were not difference from that of DW group, but it was higher in litters born from DES treated mother. No malformation of pups was found.

Group	Uterus ($x10^{-2}$)	Ovary ($x10^{-2}$)	Testis ($x10^{-2}$)	Epididymis ($x10^{-2}$)	Seminal vesicle ($x10^{-2}$)
DW	0.609±0.002	0.075±0.000	0.732±0.001	0.235±0.000	0.638±0.001
10-PM	0.542±0.001	0.072±0.000	0.715±0.001	0.241±0.000	0.619±0.001
100-PM	0.509±0.002	0.075±0.000	0.724±0.001	0.236±0.001	0.651±0.002
DES	0.469±0.010	0.066±0.001	0.650±0.000	0.257±0.000	0.522±0.000

Table 14. Relative weights of ovary and uterus in females, and testis, epididymis and seminal vesicles in males of the litters borne from mothers treated with distilled water, *P. mirifica* and diethylstilbestrol.

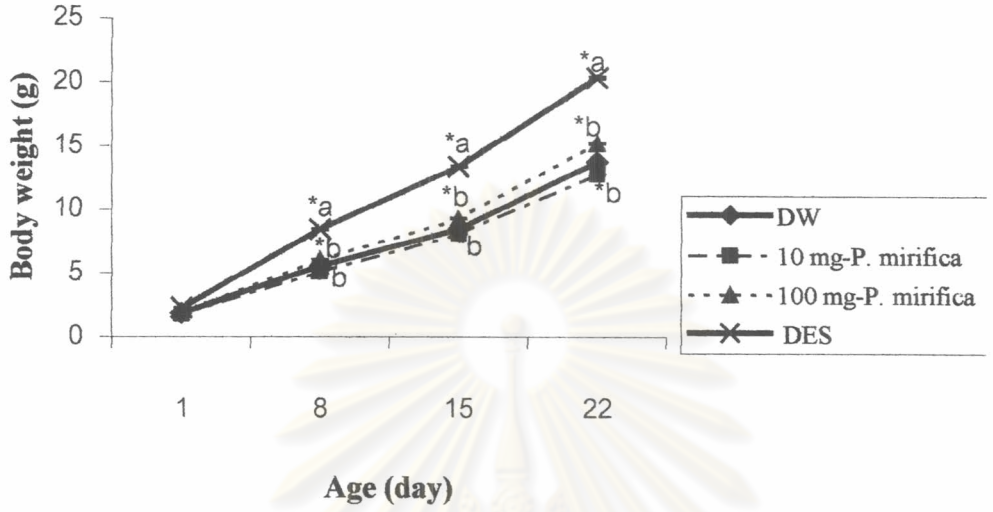


Figure 31. Post-partum growth rate of litters born from mothers treated with distilled water, *P. mirifica* and diethylstilbestrol.

* Significantly different from day-1 ($p < 0.05$)

[a] Significantly different from DW group ($p < 0.05$)

[b] Significantly different from DES group ($p < 0.05$)