CHAPTER IV

RESULTS

Experiment 1: The effects of genistein on NMU-induced tumorigenesis

The effects of genistein on NMU-induced mammary tumorigenesis

After NMU injection, the first tumors were palpable at the 34 th and 42 nd day of study period with the mean latency of 59.90 $\pm$ 2.70 and 57.30 $\pm$ 3.30 days, respectively, in vehicle and genistein groups (Table 5). No significant differences in the duration of tumor development were observed between two groups. One rat in genistein group died at the 14th day of study period after the blood collection, thus, the remaining number of rats at the end of study period is 19 rats. No death of rats (n = 20) was occurred in vehicle group. Eight rats in vehicle group and nine rats in genistein group were terminated before the 20th week whilst their tumor sizes were 3.5 cm in diameter. At the end of experiment period, all rats in both groups showed the developed tumors. The mean tumor weight in the genistein group (18.90 $\pm$ 2.50 g) tended to be higher than that of vehicle group (14.80 $\pm$ 2.00 g)($p = 0.106$) (Table 5). Female rats treated with genistein developed significantly greater tumor cross-sectional area than those of vehicle group at the 11st, 12nd, 13rd and 20th week (Figure 10A). At the end of the study period, the average tumor cross-sectional areas were 1343.59 $\pm$ 150.82 mm^2 and 1811.85 $\pm$ 203.68 mm^2 for vehicle and genistein groups, respectively (Table 5). Tumor multiplicity was also observed significantly higher in genistein group than the vehicle group at the 12nd, 13rd, 17th, 18th and 20th week of the study period (Figure 10B). Tumor multiplicities at the end of the study period were 4.65 $\pm$ 0.49 and 6.21 $\pm$ 0.75 tumors/rat for vehicle and genistein groups, respectively (Table 5). Five percent of rats (one twentieth of rats) in the vehicle group showed metastases only to the uterus, whereas 21% of rats (four nineteenth of rats) in genistein group showed metastases to the uterus (3 rats), spleen (1 rat) and liver (1 rat). One rat in genistein group showed metastases to both of uterus and liver. However, there was no significant difference in the metastases between two treatment groups ($p = 0.134$). Histological analyses of liver metastases showed the normal hepatic cords and neoplastic

cells with variation in size and shape (pleomorphism), characteristically the nuclei contain an abundance of DNA, extremely dark staining (hyperchromatin)(Figure 11). Glandular structure was also observed. Figure 12 shows metastatic cancer in spleen of genistein treated rat. Splenic pulps extensively replaced by pale neoplastic cells with large nucleoli were seen.

Table 5. Tumor parameters after vehicle and genistein treatment for 20 weeks

Treatment	Vehicle (n=20)	Genistein (n=19)
Tumor bearing rat	20	19
Tumor incidence (%)	100	100
Mean latency (days)	59.90 ± 2.70	57.30 ± 3.30
Tumor multiplicity (tumors)	4.65 ± 0.49	6.21 ± 0.75*
Tumor weight (g)	14.80 ± 2.00	18.90 ± 2.50
Tumor cross-sectional area (mm^2)	1343.59 ± 150.82	1811.85 ± 203.68*
Number of rat showing metastases	1	4
Percentage of rat showing metastases	5	21

* = significantly different from vehicle group ($p<0.05$)

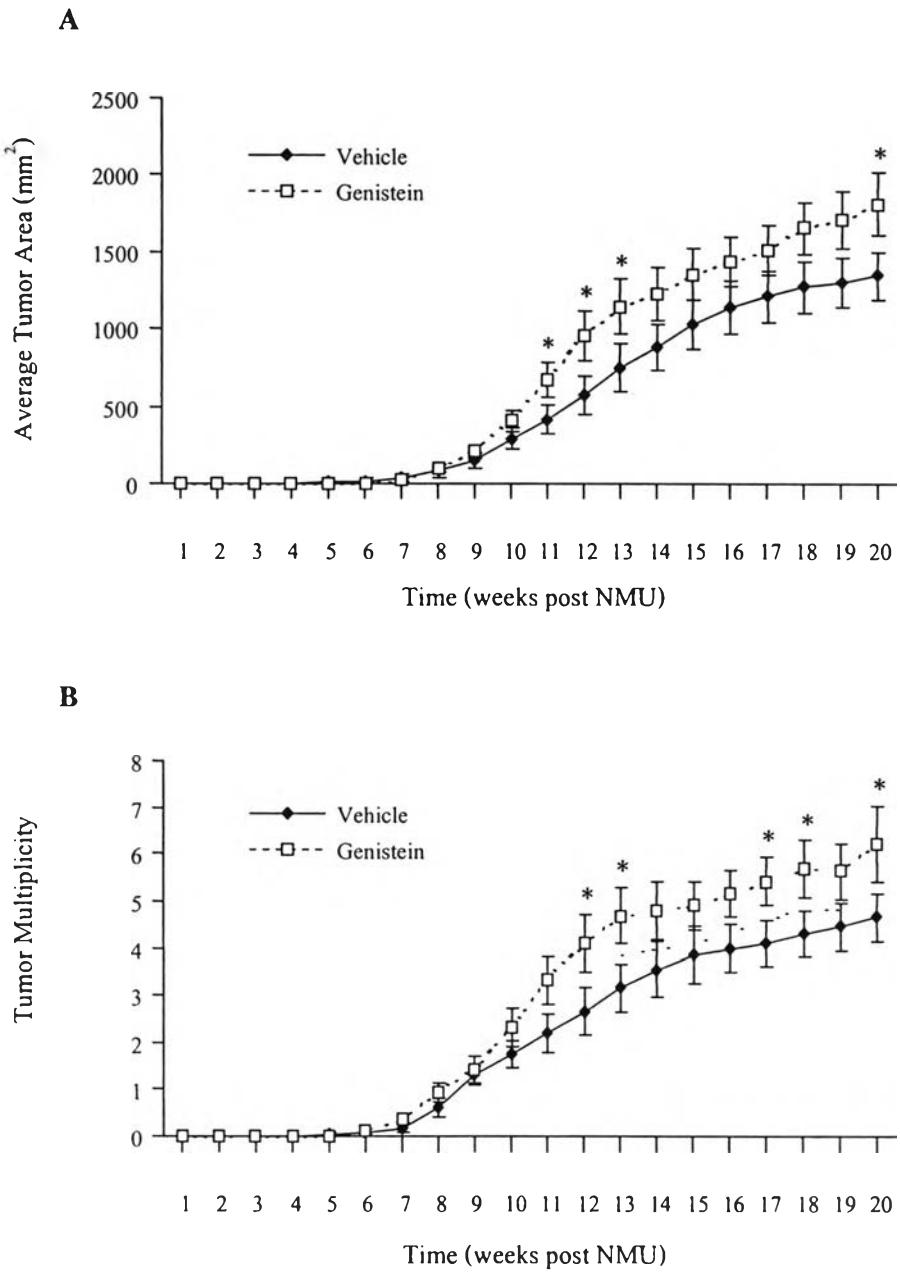

Figure 10. Tumor cross-sectional area (A) and multiplicity (B) of NMU-rats treated with 2% DMSO in peanut oil (vehicle) or genistein.

* = significantly different from vehicle group ($p<0.05$)

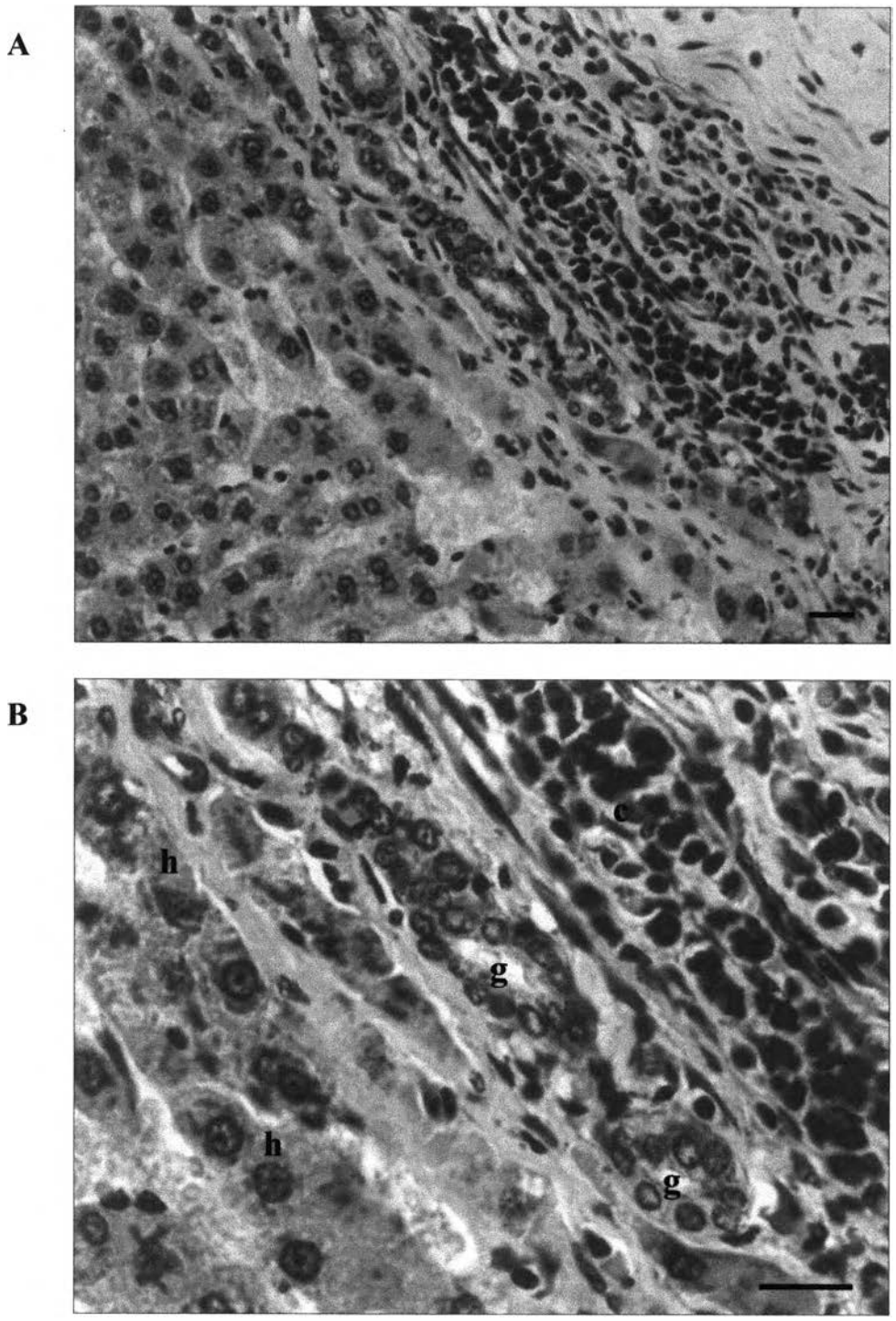

Figure 11. Metastatic cancer in liver of genistein treated NMU-rats.

g = glandular pattern, h = hepatocyte, c = cancerous cell

H&E stain, x 200 (A), x 400 (B), Bar scale = 20 μM

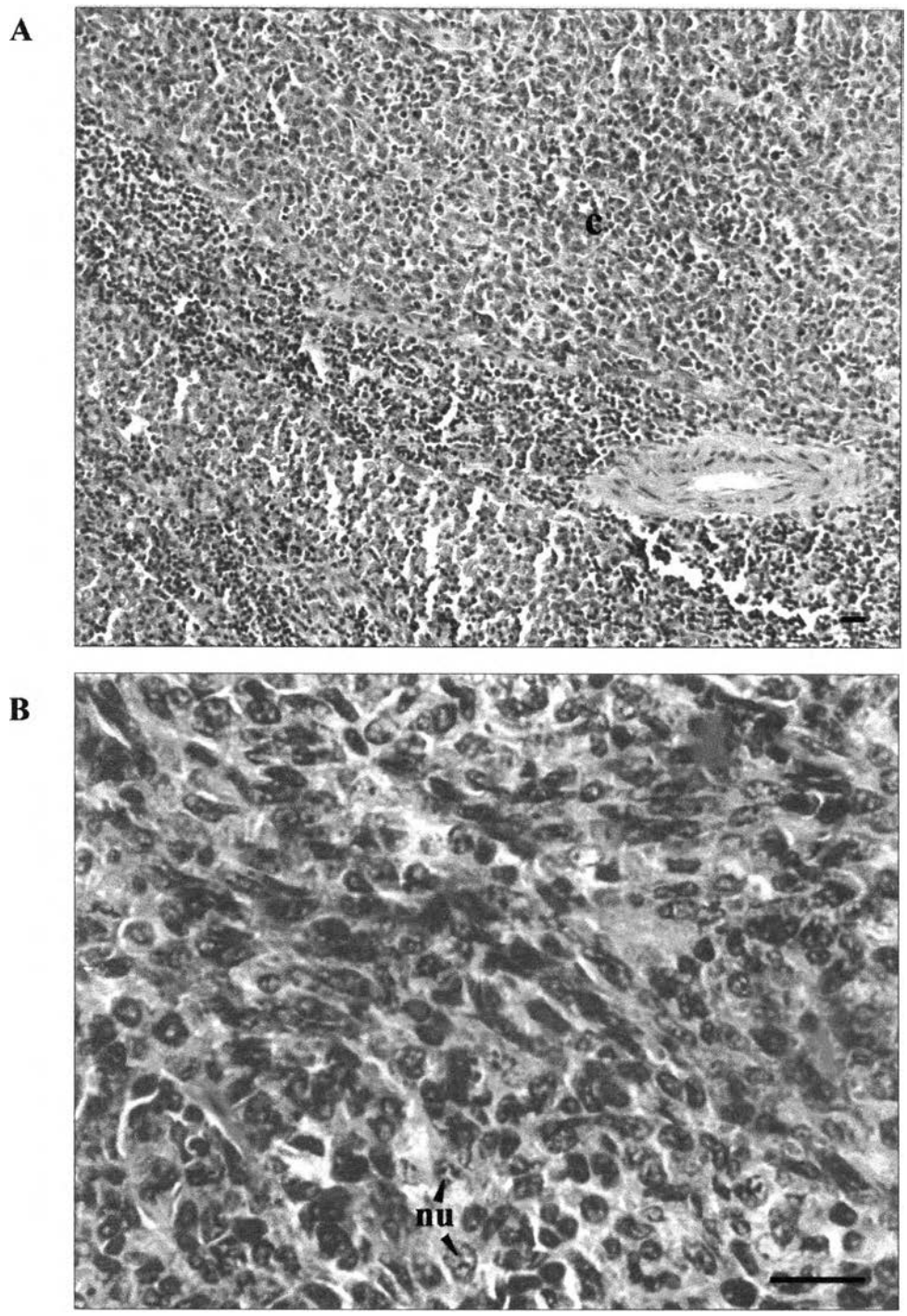

Figure 12. Metastatic cancer in spleen of genistein treated NMU-rats. Patches of large and pale cancer cells (c) with large nucleoli (nu) were shown.

H&E stain, x 100 (A), x 400 (B), Bar scale = 20 μM

Histopathological examination revealed that there are three types of mammary tumors; carcinoma, carcinosarcoma and tubular adenoma. All randomly selected mammary tumors (40 tumors) in genistein group were carcinoma type, while 38 of 40 tumor specimens in vehicle group were carcinoma type. The remaining two tumor specimens in vehicle group showed either mammary carcinosarcoma or tubular adenoma type. The histological classification of the mammary tumor type and subtype was shown in Table 6. There were no differences in mammary carcinoma subtypes between vehicle and genistein groups.

Rat mammary tumors were composed of a single histologic type or combinations of several patterns. The most mammary carcinoma subtype found in this study was papillary carcinoma. The detail in each tumor subtypes observed in vehicle and genistein groups is as follows:

Papillary carcinoma contained delicate fibrovascular cores, heavily infiltrated by lymphocytes and mast cells. The epithelium grows on top of the fibrovascular core, most of epithelial cell population was uniform in shape and size (Figure 13). Mitotic figures were found (Figure 14).

Cribriform carcinoma, the tumor which was the result of neoplastic epithelial cell proliferation in a solid pattern interrupted by round or irregularly shaped secondary lumina of variable size. The cribriform type of tumor appears in general as a uniform pattern (Figure 15), but it may be associated with papillary or comedo (Figure 16) patterns in the same tumor.

Comedo carcinoma, the tumor that was characterized by distended ductal structures lined by multilayered epithelium and centrally located necrotic debris (Figure 16).

Tubular carcinoma, the tumor that was composed of well-defined tubular or alveolar structures (Figure 17). It is postulated that tubular carcinoma originates from alveolar buds or from alveolar bud derived adenomas, whereas all the other subtypes of carcinomas described above are ductal in origin.

Carcinosarcoma, the tumor which has malignant characteristics in both the epithelium and the stroma. The epithelial component varies from well-differentiated tubular structures to poorly demarcated and elongated cells, which are difficult to differentiate from neoplastic stromal cells (Figure 18).

Adenoma was benign epithelial neoplasms that form glandular patterns and characterized by proliferation of ductular or alveolar structures arranged in clusters and separated by scant connective tissue. Individual alveoli were lined by a single layer of low cuboidal epithelial cells. The epithelial cells have small nuclei with one inconspicuous nucleolus. The lumen of the alveoli composing a tubular adenoma has a round and smooth border (Figure 19).

Table 6. Histological classification of mammary tumors.

	Vehicle (n=40)	Genistein (n=40)
Carcinoma type		
Papillary	11	14
Cribriform	3	6
Tubular	5	5
Comedo	-	-
Papillary and Cribriform	7	4
Papillary and Comedo	-	-
Papillary and Tubular	3	4
Cribriform and Comedo	3	1
Cribriform and Tubular	1	1
Tubular and Comedo	5	5
Carcinosarcoma type	1	-
Tubular adenoma type	1	-

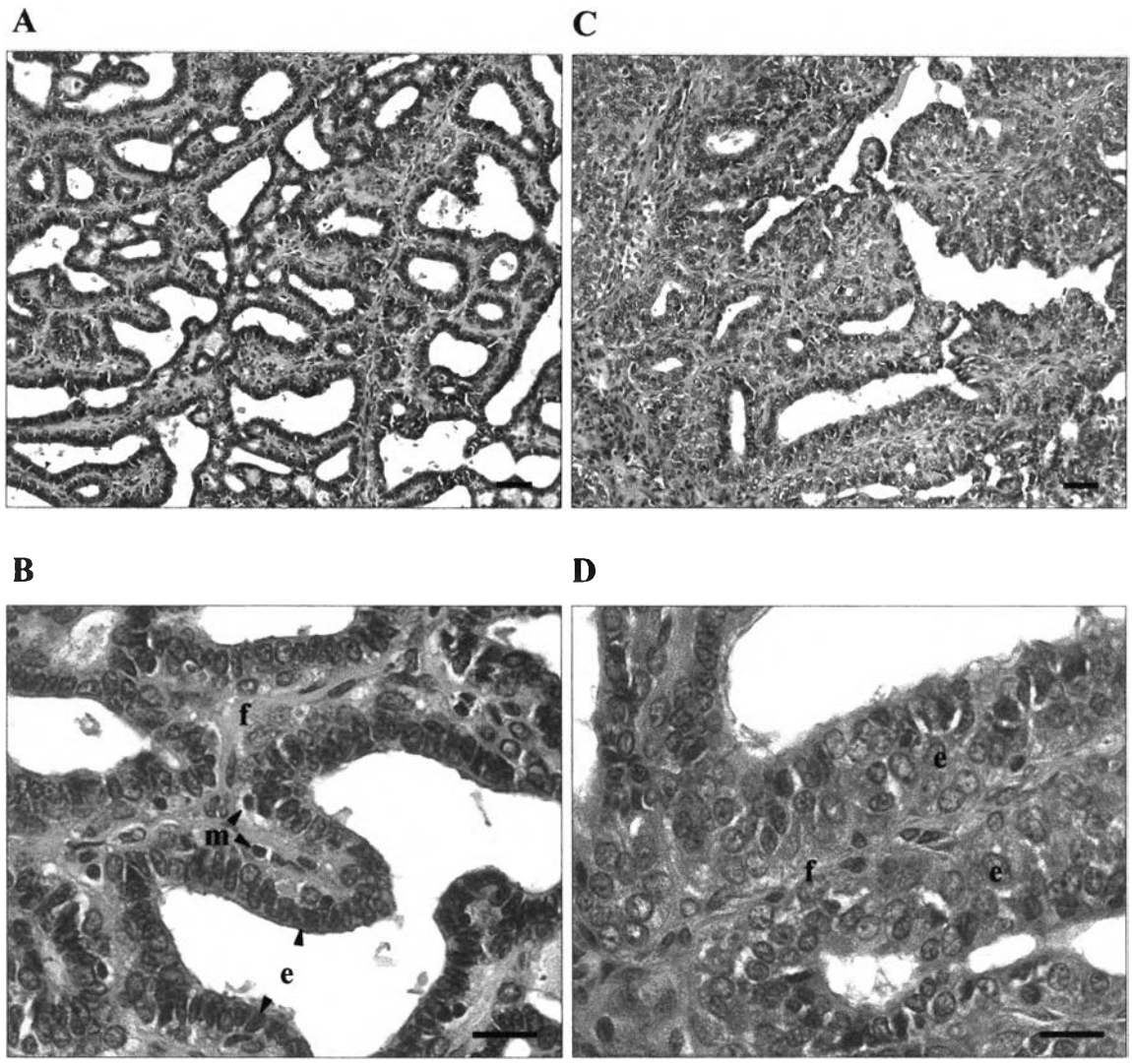

Figure 13. Papillary carcinoma of vehicle treated NMU-rat (A) and genistein treated NMU-rat (C).

H&E stain, x 100, Bar scale = 20 μM.

Papillary carcinoma with numerous papillary projections sustaining by thin connective tissue cores was seen in vehicle treated NMU-rat (B) and genistein treated NMU-rat (D).

f = fibrovascular core, m = mast cell, e= epithelium

H&E stain, x 400, Bar scale = 20 μM.

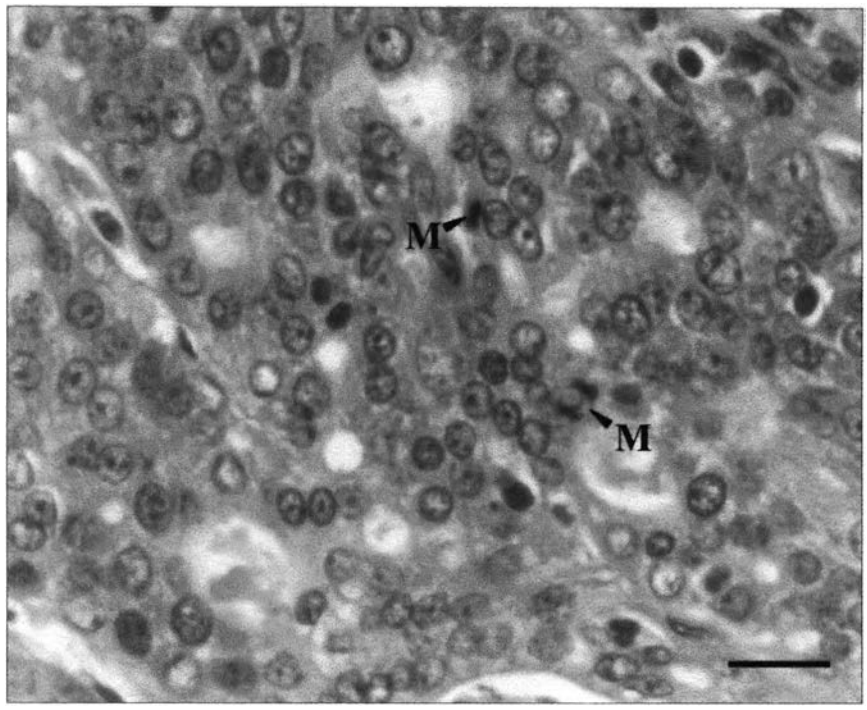

Figure 14. The number of mitoses is generally high in carcinoma.

M = mitotic figure

H&E stain, x 400, Bar scale = 20μM.

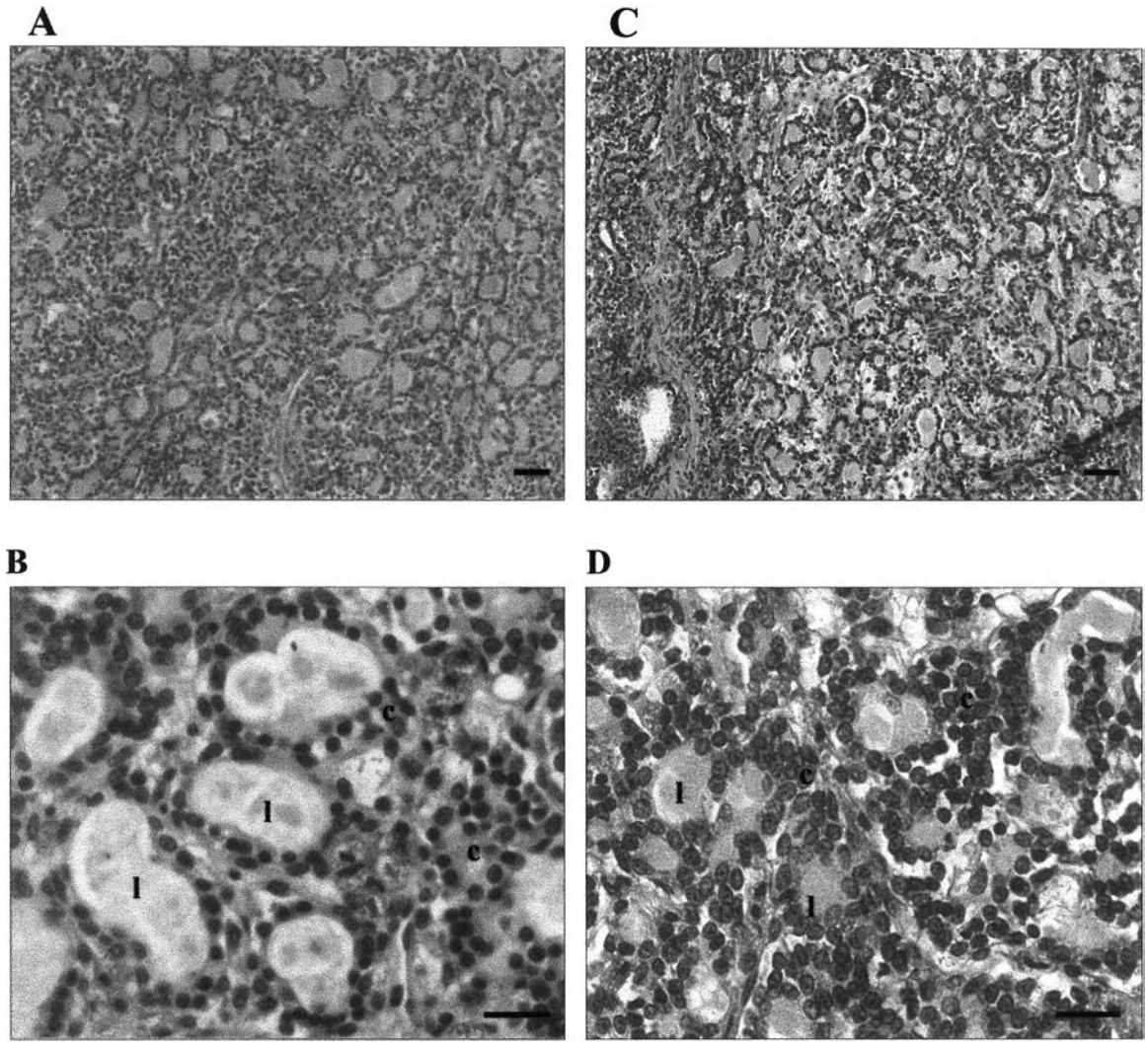

Figure 15. Cribriform carcinoma of vehicle treated NMU-rat (A) and genistein treated NMU-rat (C).

H&E stain, x 100, Bar scale = 20 μM.

Cribriform carcinoma with the solid sheets of neoplastic epithelial cells were interrupted by round or irregularly shaped secretory lumina of variable size of vehicle treated NMU-rat (B) and genistein treated NMU-rat (D).

l = secretory lumina, c = cancer cell

H&E stain, x 400, Bar scale = 20 μM.

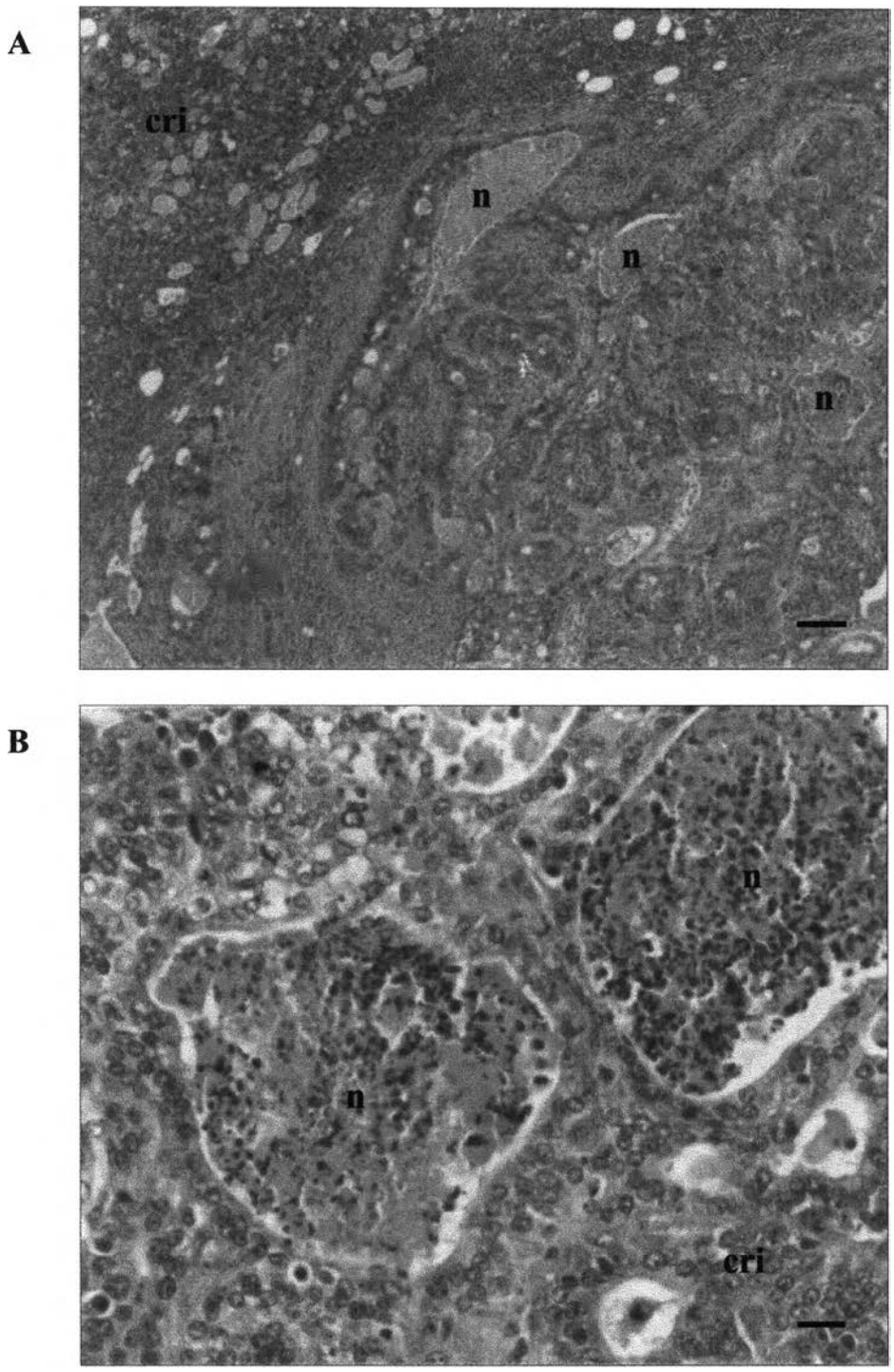

Figure 16. Cribriform and Comedo carcinomas of vehicle treated NMU-rat (A).

H&E stain, x 40, Bar scale = 100 μM.

Cribriform and Comedo carcinoma (B).

n = necrotic area, cri = cribriform pattern

H&E stain, x 200, Bar scale = 20 μM.

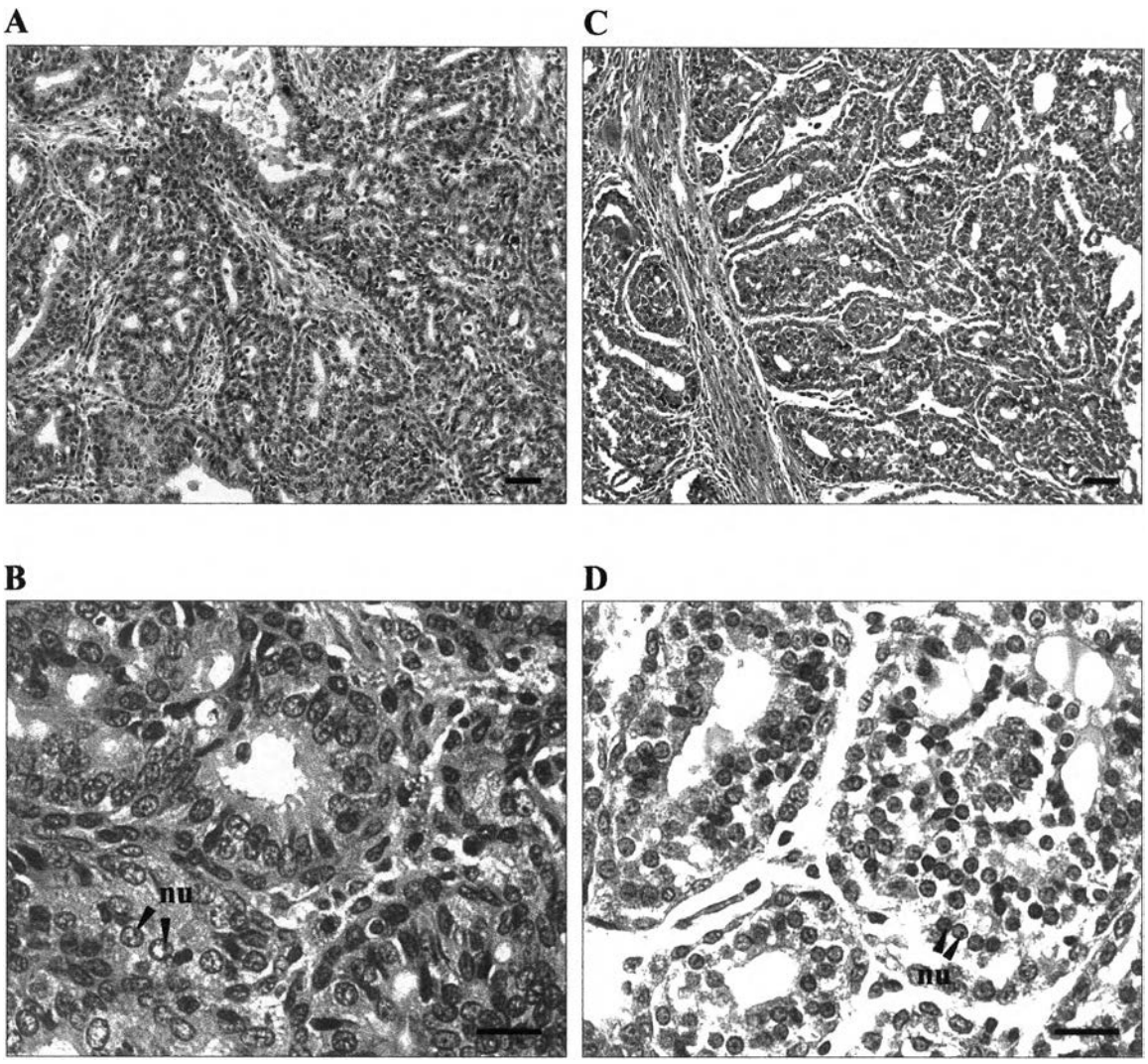

Figure 17. Tubular carcinoma of vehicle treated NMU-rat (A) and genistein treated NMU-rat (C).

H&E stain, x 100, Bar scale = 20 μM.

Tubular carcinoma composed of well-defined tubular or alveolar structures. The epithelial cells composing the tubular carcinoma had increased nuclear size and contained prominent nuclei (nu) of vehicle treated NMU-rat (B) and genistein treated NMU-rat (D).

H&E stain, x 400, Bar scale = 20 μM.

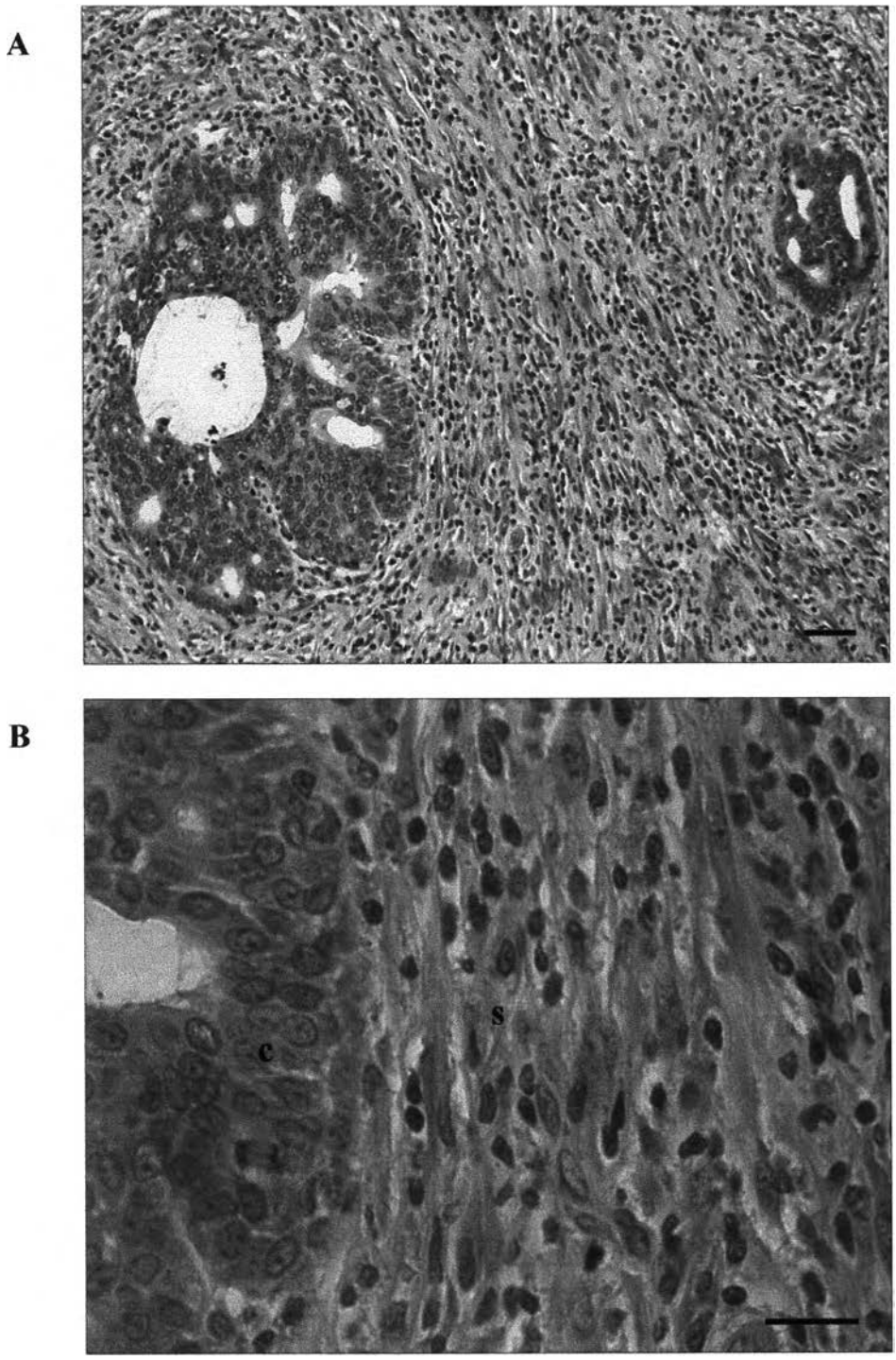

Figure 18. Carcinosarcoma of vehicle treated NMU-rat (A).

H&E stain, x 100, Bar scale = 20μM.

Carcinosarcoma with malignant characteristics in both the epithelium and stromal parts (B).

c = carcinoma, s = sarcoma

H&E stain, x 400, Bar scale = 20μM.

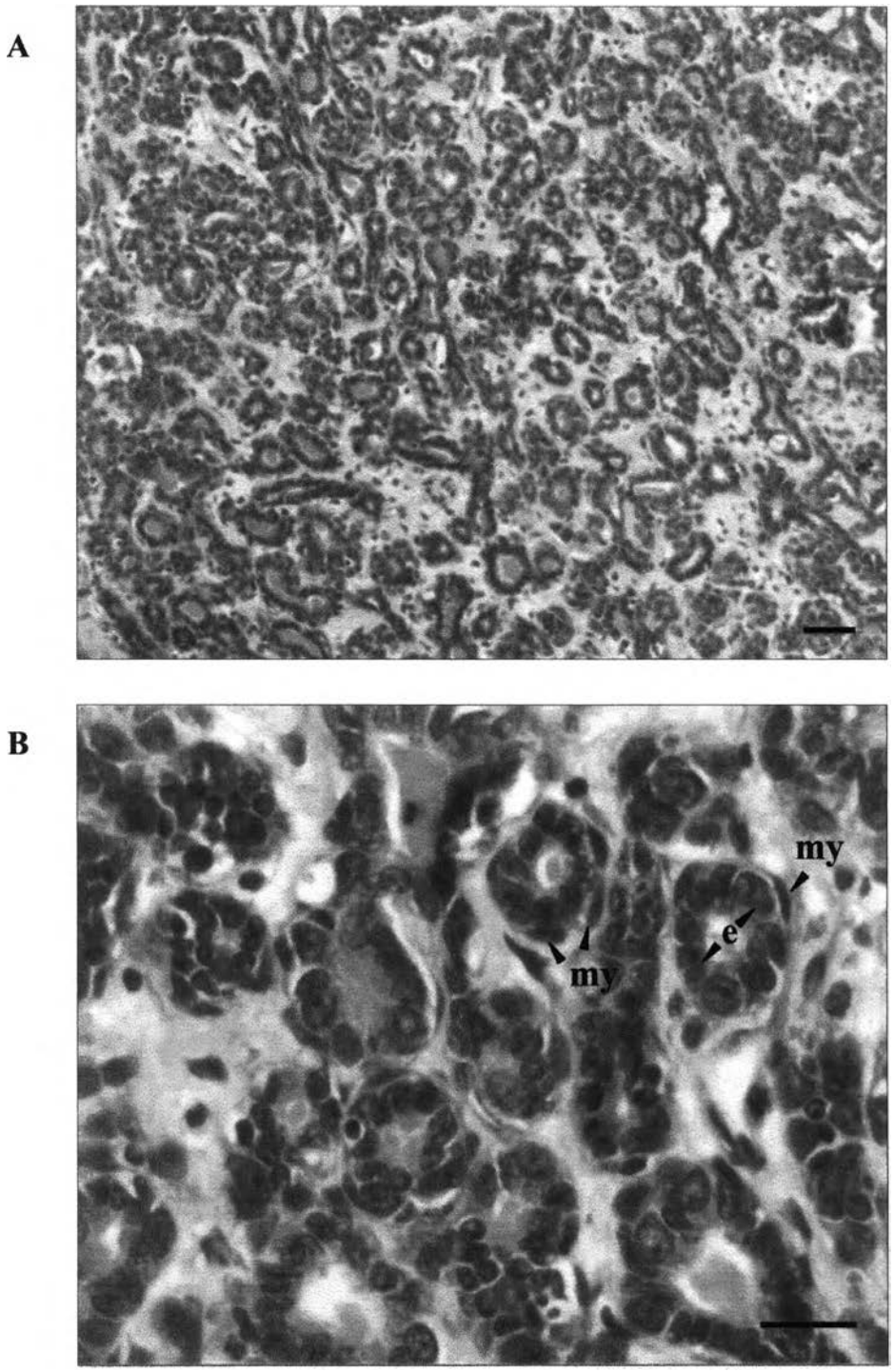

Figure 19. Tubular adenoma of vehicle treated NMU-rat (A) and genistein treated NMU-rat (B). H&E stain, x 100, Bar scale = 20μM.

Tubular adenoma with the number of acini was increased. The shape of individual acini and the component histological parts remained normal. The alveoli exhibited a regular contour. Individual alveolar was composed of one layer of secretory epithelium (e). Myoepithelial cells (my) were present. (B). H&E stain, x 400, Bar scale = 20μM.

Diet intake and body and organ weights of rats

Average diet intakes of rats treated with genistein did not difference from that of vehicle group throughout the study period (approximately 13-15 g/rat/day) (Figure 20). The mean body weights of rats in vehicle and genistein groups are shown in Figure 21 and Table 7. There were no significant differences of body weights between these two groups throughout the study period. The absolute and relative weights of livers, uteri and ovaries were shown in Table 7, which were not significantly different between the genistein and vehicle groups.

Effect of genistein treatment on estrous cycle

After the analysis, it was found that the estrous cycle lengths in rats were gradually changed, month by month. Thus, the estrous cycle lengths were monthly pooled and calculated. Vaginal smears taken during the study period indicated that all rats of both vehicle and genistein groups showed regular estrous cycles of 4 to 5 days during the first few months: 3 months in the vehicle group and 2 months in the genistein group (Table 8). The estrous cycle lengths were significantly increased ($p < 0.05$) thereafter in both groups when compared to the first month (approximately 6-7 days/cycle). There was no significant difference on the estrous cycle length between two groups.

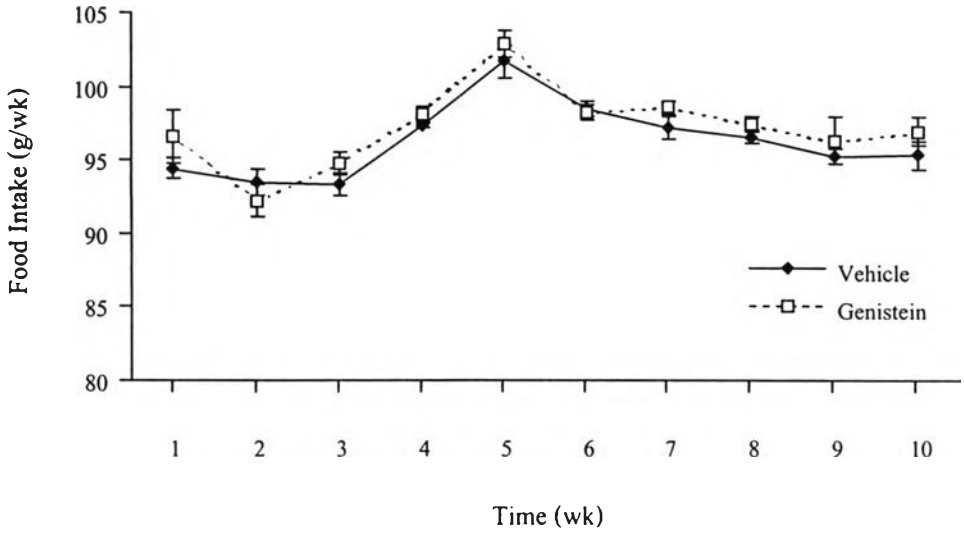

Figure 20. The diet intake of NMU-rats treated with vehicle or genistein

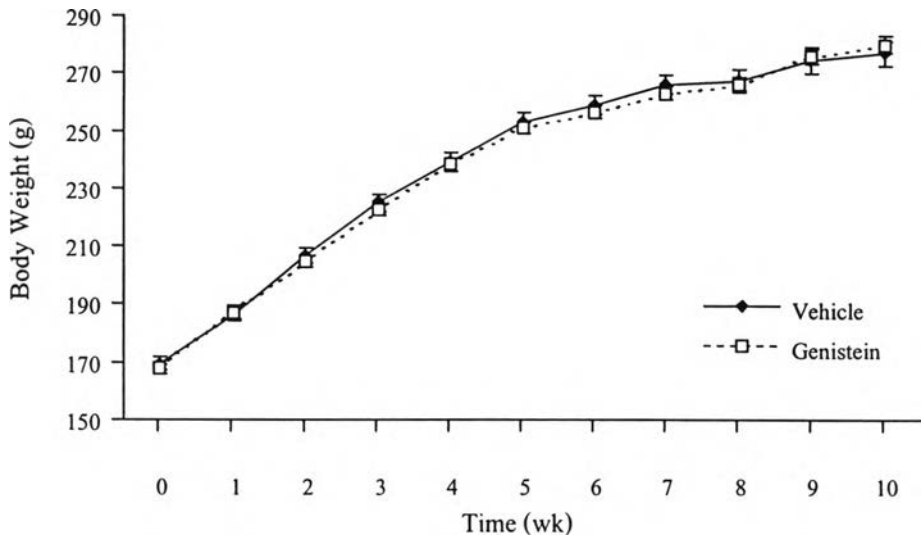

Figure 21. The body weights of NMU-rats treated with vehicle or genistein.

Table 7. Body weights and absolute and relative organ weights of vehicle and genistein treated NMU-rats.

Treatment	Vehicle (n=20)	Genistein (n=19)
Body weight (g)		
1 week	169.5 ± 1.7	167.6 ± 2.1
20 week	292.2 ± 4.8	292.0 ± 4.5
Absolute liver weight (g)	9.2 ± 0.6	9.6 ± 0.3
Relative liver weight (g/100 g BW)	3.1 ± 0.2	3.3 ± 0.1
Absolute uterine weight (mg)	459.2 ± 30.1	495.4 ± 37.5
Relative uterine weight (mg/100g BW)	154.4 ± 9.7	167.6 ± 12.3
Absolute ovarian weight (mg)	129.4 ± 6.6	124.1 ± 4.7
Relative ovarian weight (mg/100g BW)	43.7 ± 2.1	42.4 ± 2.6

Relative weights of all organs are measured in g (or mg)/100 g of BW.

Table 8. Estrous cycle lengths (mean ± SE) during 5 months of study period in NMU-rats treated with vehicle or genistein.

Group	Estrous cycle length (day)				
	M_1	M_2	M_3	M_4	M_5
Vehicle	4.67 ± 0.07	4.92 ± 0.08	5.64 ± 0.30	6.14 ± 0.61*	6.47 ± 0.93*,+
Genistein	5.07 ± 0.11	5.06 ± 0.17	6.47 ± 0.58*	7.13 ± 0.68*,+	7.05 ± 1.13*,+

M_1 to M_5 = The first to the fifth month of study period

* = significantly different from M_1 in each group ($p<0.05$)

+ = significantly different from M_2 in each group ($p<0.05$)

Effect of genistein treatment on serum estradiol concentration

Changes of serum estradiol levels in adult female rats treated with genistein or vehicle were shown in Figure 22. The serum estradiol concentrations at the first day of experiment (0 month) were not significantly different between the vehicle and genistein groups (33.9 ± 1.9 and 33.5 ± 1.6 pg/ml, respectively). Compared to the first day of study period, serum E_2 levels in both genistein and vehicle groups significantly decreased at the first month and then recovered to the basal level at the second month and kept no changes throughout the experimental period. Serum E_2 levels in rat treated with genistein was significantly higher than those of vehicle group at the first and second month of treatment period.

Serum genistein concentration in vehicle and genistein treated rats

At the end of experiment period, serum samples were randomly selected from 13 rats in each group of vehicle and genistein treatment, and determined free genistein levels. Free genistein levels were varied individually; however, there was no significant difference between the two treatment groups, 0.18 ± 0.03 μg/ml and 0.19 ± 0.02 μg/ml in vehicle and genistein groups, respectively (Figure 23A). Unluckily, determination of free genistein concentration was consumed almost all of the serum samples collected (300 μl), only some samples were remained enough for the next total genistein concentration determination. Therefore, the total genistein concentration was assessed in only five rats each from vehicle and genistein groups. The five serum samples were selected randomly and they seemed to be a good representative of all samples, because rats with various levels of free genistein (high, medium and low) were chosen (rat number: V13, V44, V33, V11 and V9 of vehicle and G48, G20, G38, G22 and G15 of genistein group). Average free genistein concentrations in the five serum samples selected were not significant different between genistein and vehicle treated rats (0.47 ± 0.07 μg/ml and 0.33 ± 0.03 μg/ml), respectively. The total genistein concentration was significantly increased in the genistein treated group (1.34 ± 0.06 μg/ml) when compared with the vehicle treated group (0.75 ± 0.18 μg/ml) (mean ± SE)($p<0.01$) (Figure 23B).

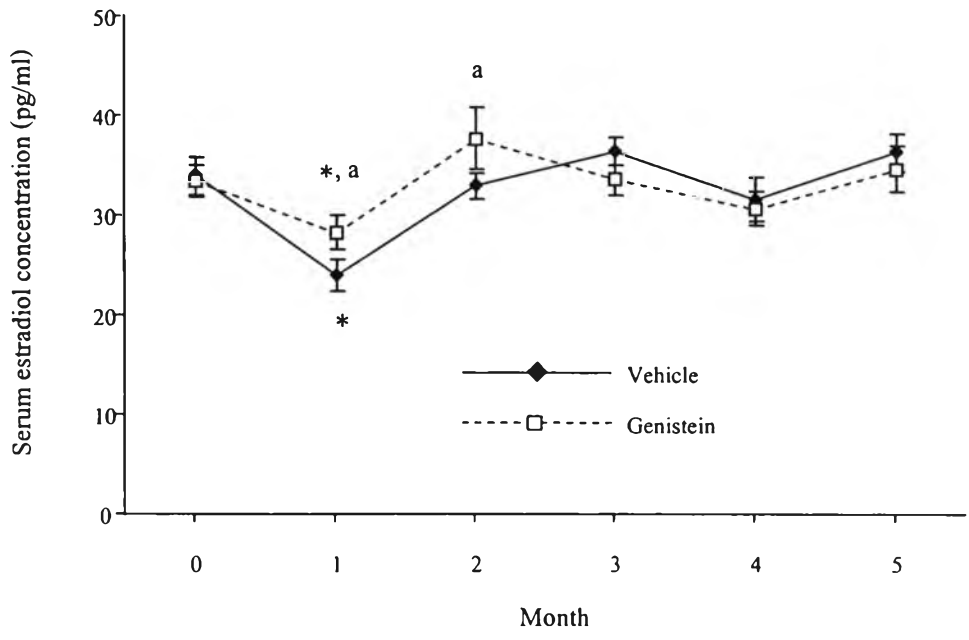

Figure 22. Changes of serum estradiol levels in NMU-rats treated with vehicle or genistein.

a = significantly different from vehicle group ($p<0.05$)

* = significantly different from 0 month ($p<0.05$)

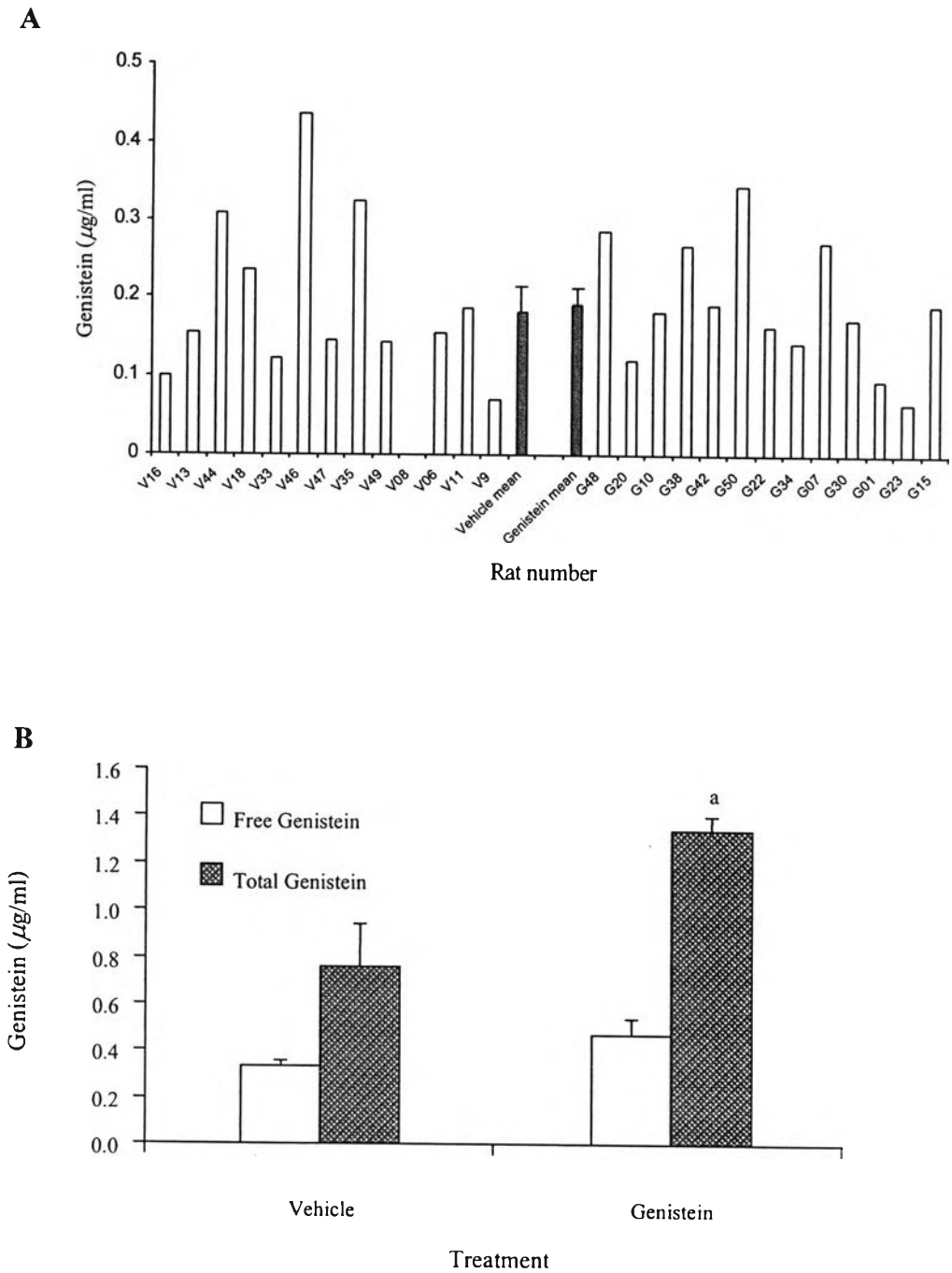

Figure 23. Serum free and total genistein concentrations in NMU-rats treated with vehicle or genistein.

a = significantly different from vehicle group ($p<0.05$)

V_x and G_x = rats (in "X" number) treated with vehicle and genistein.

Experiment 2: The effects and mechanism of genistein and tamoxifen on NMU-induced mammary tumor growth

The effect of tamoxifen and genistein on mammary tumor growth

After the tumor reached a 1-cm diameter (approximately after 8-10 weeks of NMU injection), rats were treated with vehicle (2% DMSO in peanut oil), genistein, tamoxifen and tamoxifen plus genistein for 10 weeks. In vehicle group, the average cross - sectional area of tumor continuously increased until reaching the size of approximately 800 mm^2 of 6 wk of treatment and it remained at this size until the end of experiment. The average cross - sectional area of tumor in genistein group continucusly increased throughout the experimental period. The size was 1,081 $\pm$ 116 mm^2 at the end of treatment which tended to be larger than that of vehicle group (776 $\pm$ 227 mm^2)(P=0.15). Tumor cross-sectional area in tamoxifen treatment alone or in combination with genistein gradually increased and reached the size of approximately 300 mm^2 at 5 wk of treatments and remained at this size until the end of experiment. The tumor sizes were significantly smaller than those of vehicle and genistein groups ($p< 0.05$)(Figure 24).

The tumor multiplicities of rats are shown in Figure 25. The vehicle and genistein treated rats showed a gradual increase in tumor multiplicity throughout the treatment period. It was 2.2 $\pm$ 0.5 tumors/rat in vehicle group and 2.2 $\pm$ 0.2 tumors/rat in genistein group, respectively, at the end of study period. On the other hand, tumor multiplicity in tamoxifen treated groups was significantly lower than those of vehicle and genistein groups after 3 wk of treatment. Treatment of genistein together with tamoxifen was diluted the suppressive effect of tamoxifen on the tumor multiplicity, starting from the 5^{th} week. Since the tumor multiplicity in tamoxifen plus genistein group was significantly lower than that of vehicle and genistein treated groups only at the third and fourth week of treatment.

The mean tumor weights of rats at the end of study period are shown in Figure 26. Tamoxifen plus genistein, but not tamoxifen only, significantly ($p< 0.05$) reduced the mean tumor weights compared to the vehicle group. However, tamoxifen treatment only tended to lower tumor weight ($p = 0.107$). The mean tumor weight of genistein group was slightly higher than

that of vehicle group (13.8 $\pm$ 1.9 and 9.14 $\pm$ 2.9, respectively) ($p = 0.121$), but significantly higher than both of tamoxifen groups.

Fifty percent of rats (five of ten rats) in the genistein group showed metastases to the liver (2 rats) and uterus (3 rats), whereas 10% of rats (one of ten rats) in vehicle and tamoxifen plus genistein groups developed metastases to the uterus (1 rat) and liver (1 rat), respectively, while there was no occurred metastases in tamoxifen group (Figure 27).

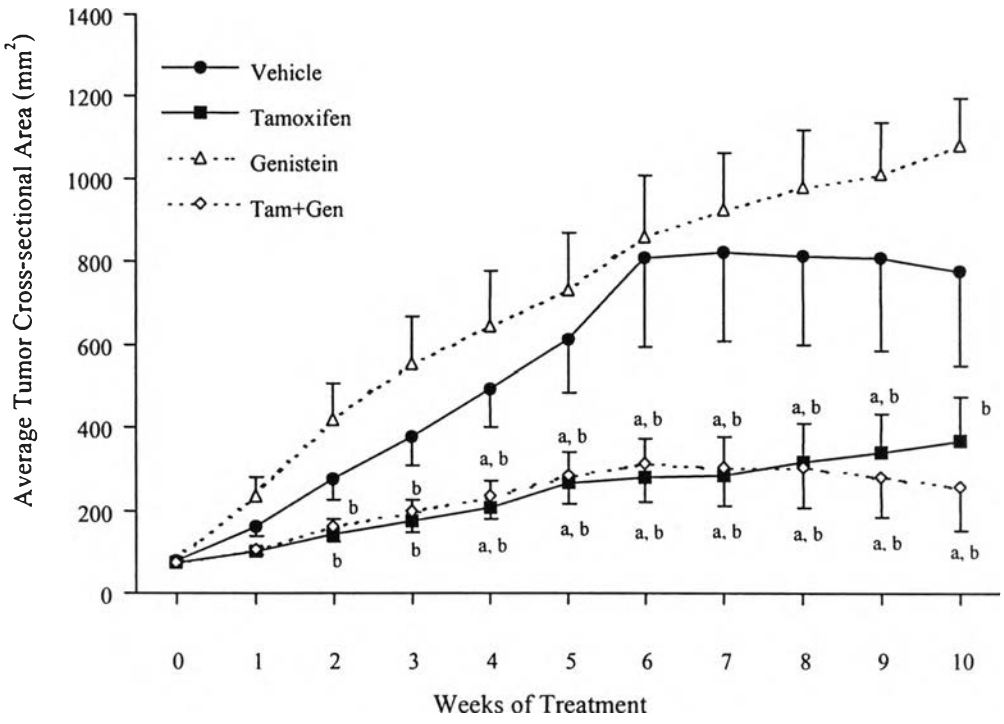

Figure 24. Tumor cross-sectional area of NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein. Treatment was started when the tumor diameter was 1 cm.

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

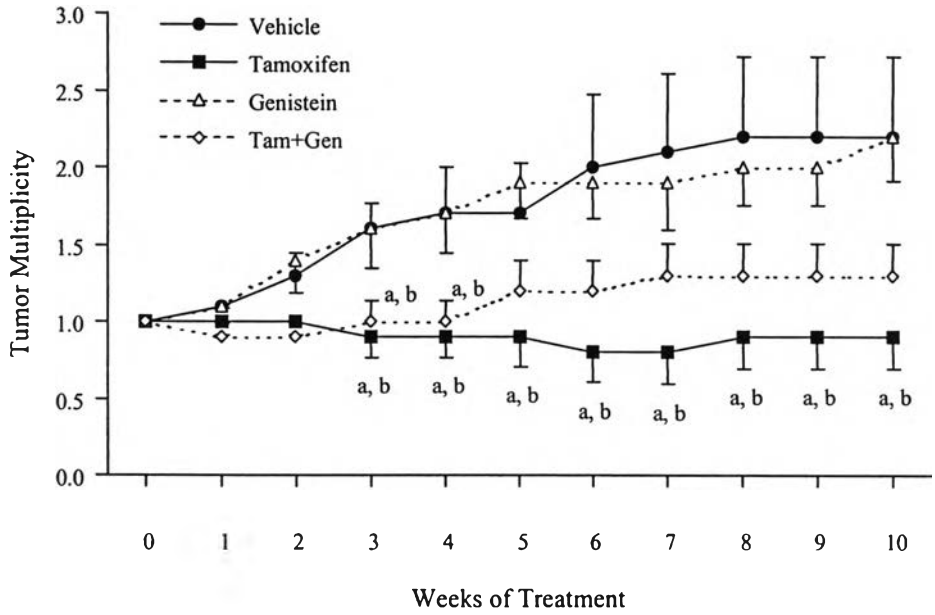

Figure 25. Tumor multiplicity of NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein. Treatment was started when the tumor diameter was 1 cm.

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

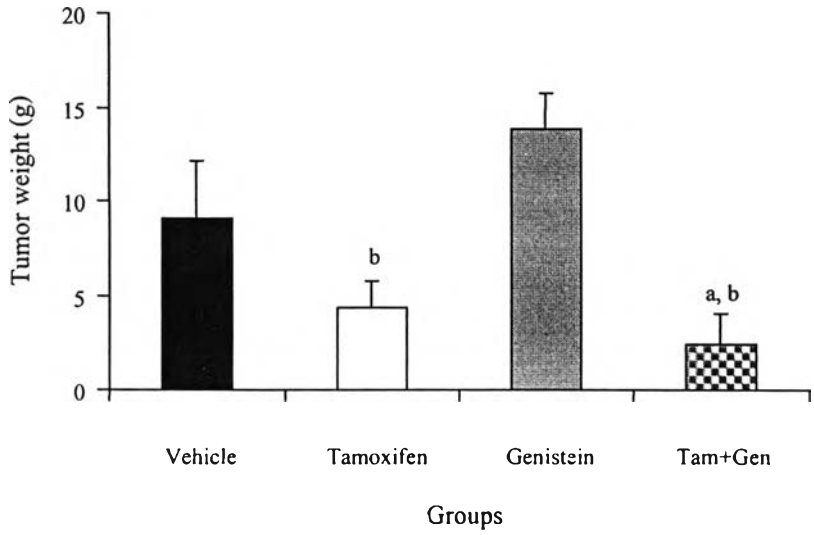

Figure 26. Tumor weights of NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period.

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

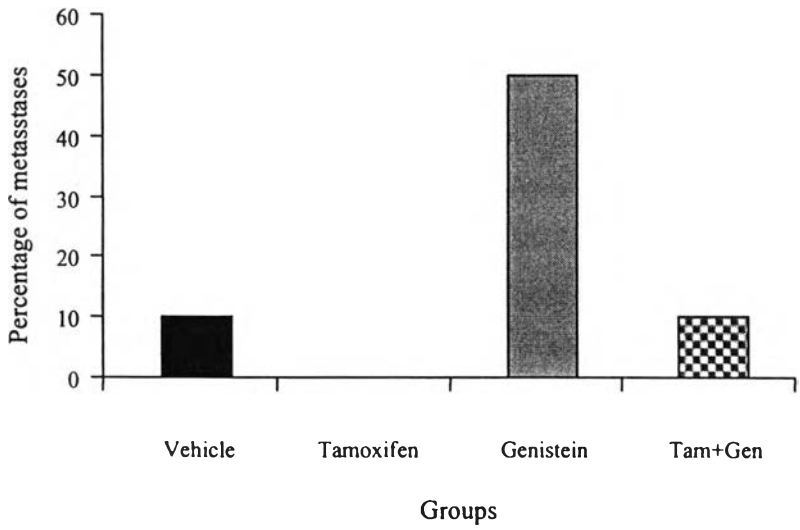

Figure 27. Percent metastases of NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period.

The mechanism of action of tamoxifen and genistein on mammary tumor growth

Expression of genes related to estrogen receptor pathway

Since the NMU-induced tumorigenesis has been described to be estrogen receptor (ER) dependent, ERα, ERβ and pS2 gene expressions were determined in the mammary tumors and then caribrated with GAPDH gene expression (house keeping gene). In the control (vehicle only) group the average expression level of ERα mRNA was 2.26 $\pm$ 0.23 folds greater than that of GAPDH. All three treatments (tamoxifen, genistein, or tamoxifen plus genistein) resulted in a significant reduction of ERα mRNA levels, but to varying extents. Thus genistein alone reduced the ERα / GAPDH to 1.58 $\pm$ 0.40 folds compared to the statistically significantly greater reduction level seen in tamoxifen (0.28 $\pm$ 0.16) or tamoxifen plus genistein (0.39 $\pm$ 0.21) (Figure 28)

Contrary to the expression levels of ERα mRNA, the expression of ERβ mRNA was very low being mostly undetectable in the first round of PCR, and only one band of nested PCR in the control group being detected (Figure 29).

With respect to pS2 mRNA expression levels, these were similar to the standard GAPDH in the control group of tumors (1.00 $\pm$ 0.12) (Figure 30), with a slight but not statistically significant elevation (1.14 $\pm$ 0.39) or depression (0.52 $\pm$ 0.20), respectively, in mRNA levels noted in the genistein and tamoxifen treatment groups. In contrast, administration of both tamoxifen and genistein together significantly reduced (0.36 $\pm$ 0.19) the relative expression level of pS2 mRNA (Figure 30). However, noted that with the high level of intra-sample variance and small sample sizes, it remains plausible that tamoxifen treatment alone is indeed similar to tamoxifen plus genistein in reducing pS2 mRNA levels and this requires further evaluation.

Expression level of genes related to growth factor signaling pathway

Neu and IGF-1 mRNA expression were detected in tumors obtained from all experimental groups. Although tamoxifen, in contrast to genistein, appeared to reduce *neu* mRNA expression levels either alone (0.31 $\pm$ 0.18) or in combination with genistein (0.60 $\pm$ 0.34) neither were statistically significant (Figure 31). Again, due to the relatively large intra-sample

variance compared to the low sample sizes analysed, it is not clear if in fact tamoxifen and tamoxifen plus genistein treatment actually do reduce relative *neu* mRNA levels (Figure 31).

In contrast, IGF-1 mRNA expression levels were significantly increased (approximately 3 folds) after treatment of genistein alone, although the co-treatment of genistein and tamoxifen was statically significantly less elevated (approximately 2 folds), perhaps suggesting some inhibition by tamoxifen although the slight decrease in IGF-1 mRNA levels seen by tamoxifen treatment alone is not significant (Figure 32).

Expression of gene related to metastasis suppressor

Direct RT-PCR amplification of GPR54 mRNA resulted in no detectable amplicons and thus two stage nested PCR amplification of the cDNA was employed. Three of the five tumors obtained from the control group yielded detectable amplicons, while one of the five was positive for both tamoxifen and tamoxifen plus genistein treatment groups. Only one very faint band occurred in the genistein group (Figure 33).

The relation of percent metastases of tumor in rats and the levels of GPR54 expression of mammary tumor was shown in Table 9. There was a relationship between GPR54 expression and percent metastases of tumors to the liver and uterus. The high percentage of metastases, in genistein group, correlated with the absence of GPR54 expression in tumor tissues.

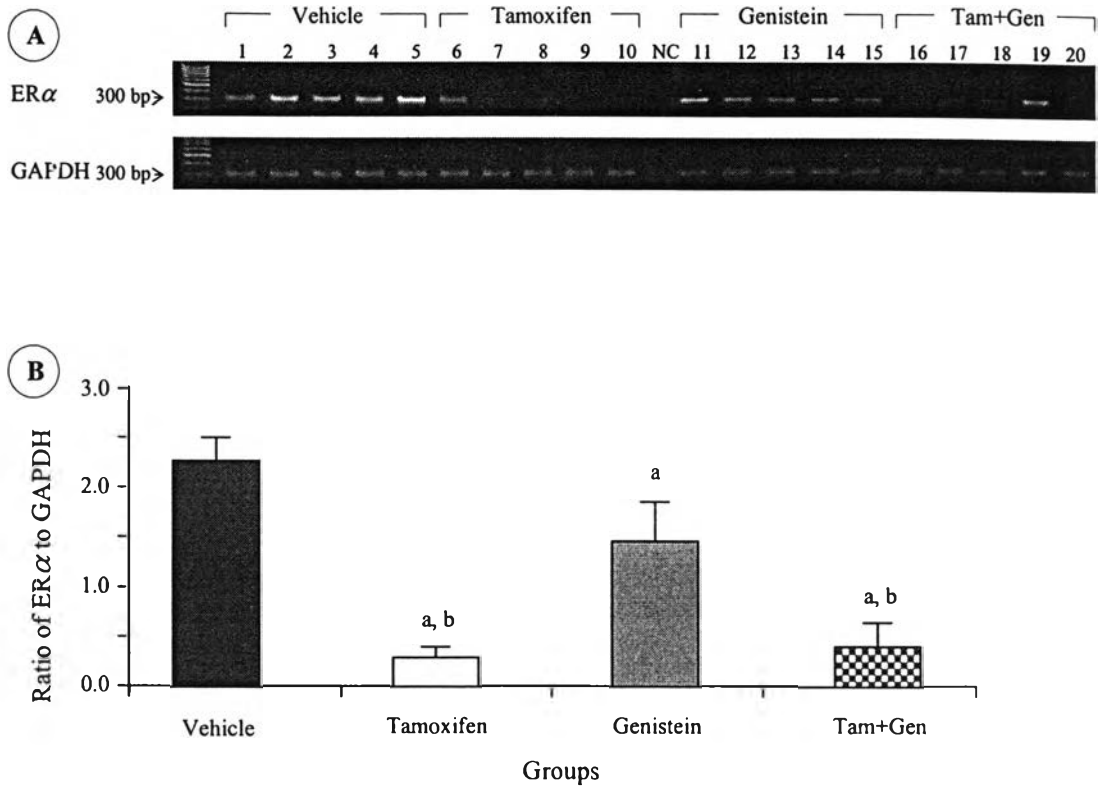

Figure 28. Semi-quantitative RT-PCR of ERα mRNA. The electrophoresed RT-PCR results (A) and the relative levels of ERα to GADPH mRNA (n =15) (B).

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

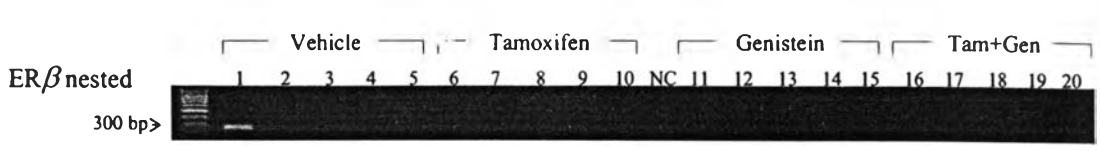

Figure 29. The nested RT-PCR result of ERβ mRNA.

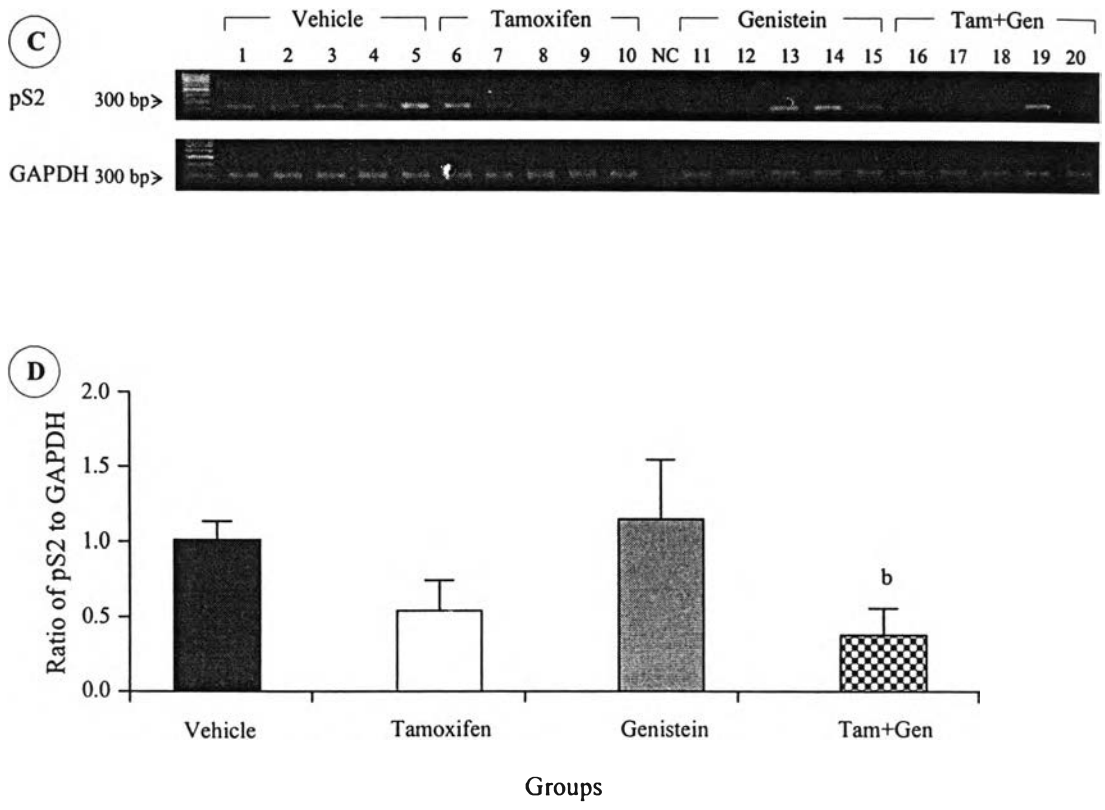

Figure 30. Semi-quantitative RT-PCR of pS2 mRNA. The electrophoresed RT-PCR results (C) and the relative levels of pS2 to GADPH mRNA (n =15) (D).

b = significantly different from genistein group ($p<0.05$)

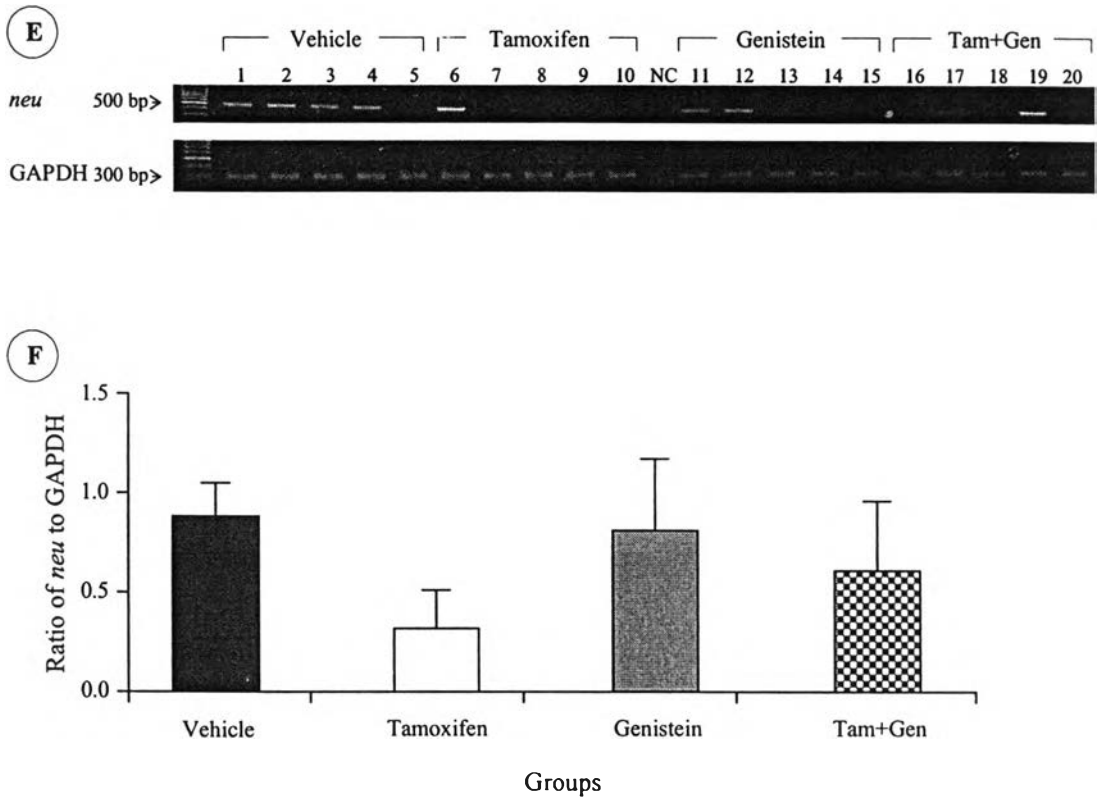

Figure 31. Semi-quantitative RT-PCR of *neu* mRNA. The electrophoresed RT-PCR results (E) and the relative levels of *neu* to GADPH mRNA (n =15) (F).

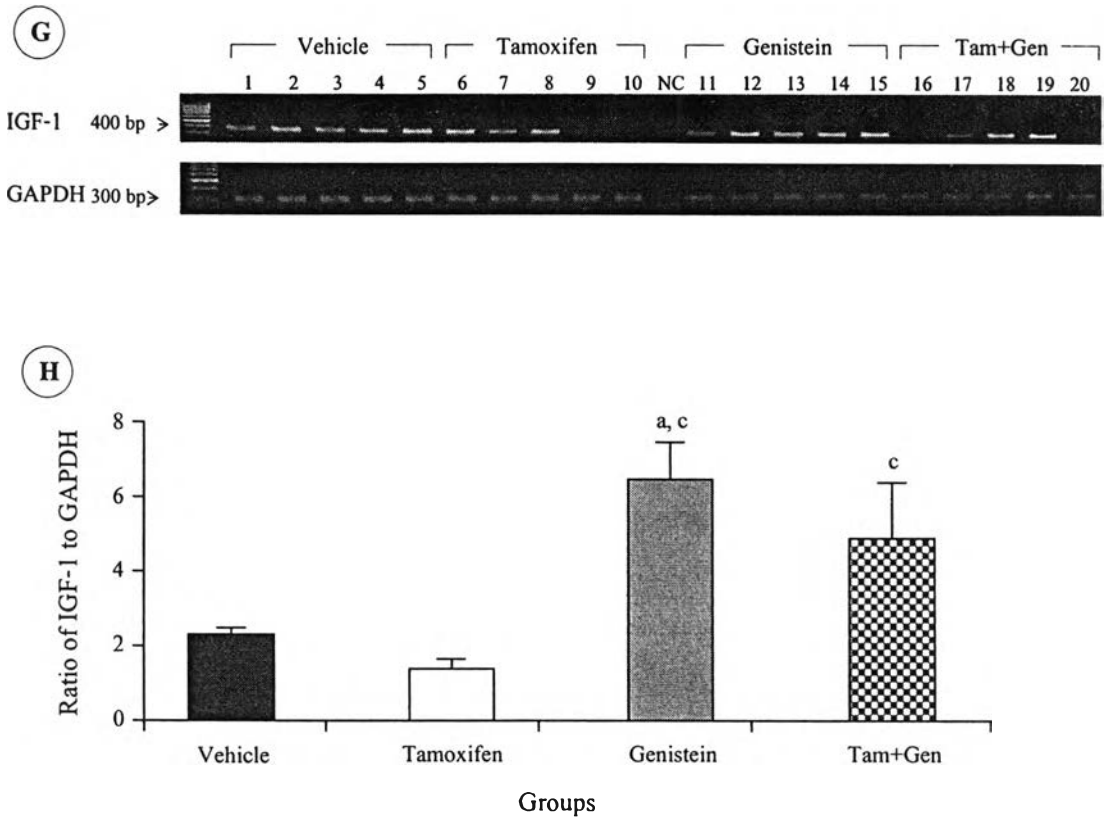

Figure 32. Semi-quantitative RT-PCR of IGF-1 mRNA. The electrophoresed RT-PCR results (G) and the relative levels of IGF-1 to GADPH mRNA (n =15) (H).

a = significantly different from vehicle group ($p<0.05$)

c = significantly different from tamoxifen group ($p<0.05$)

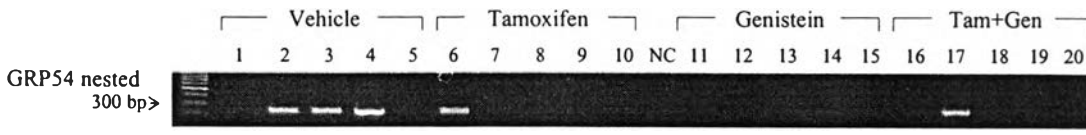

Figure 33. The nested RT-PCR result of GPR54 mRNA.

Table 9. Relation of percentage of metastases to the whole body and levels of GPR54 expression of mammary tumor.

Groups	Percentage of metastases	GPR54 expression
Vehicle	Low (10%)	+++
Tamoxifen	-	+
Genistein	high (50%)	-
Tam+Gen	low (10%)	+

Effect of genistein and tamoxifen on serum estradiol concentration

The serum estradiol concentration in rats treated with genistein and tamoxifen for 10 weeks were shown in Figure 34. There were no significantly differences in the serum E_2 levels between vehicle and genistein groups (30.33 $\pm$ 2.47 pg/ml and 26.22 $\pm$ 2.53 pg/ml, respectively). Tamoxifen and tamoxifen plus genistein treatments resulted in increase serum E_2 levels, but only tamoxifen plus genistein showed the statistically significant higher (43 $\pm$ 2.44 pg/ml)($p<0.05$). However, both of tamoxifen treatment groups were significantly higher than the genistein group.

Effect of genistein and tamoxifen on weights of liver, ovary and uterus.

Table 10 shows the results of genistein and tamoxifen treatment on body and organ weights. The body weights, the absolute and relative weights of liver, ovary and uterus of rats in genistein group were not significantly different from the vehicle group. Body weights of rats treated with tamoxifen alone or in combination with genistein were lowered than the vehicle group, but not significant different from the genistein group. Both of the absolute and relative weights of liver, ovary and uterus in rats treated with tamoxifen and tamoxifen plus genistein were significantly decreased when compared with vehicle and genistein groups ($p<0.05$).

The histological examination of liver in all four groups did not showed indications of test substance induced changes (Figure 35).

The histological study of uterus in vehicle and genistein groups showed the normal structure with a simple cuboidal epithelium cells overlying the thick lamina propria of the endometrium. The uterine glands were dispersed in their lamina propria (Figure 36). In contrast, rats treated with tamoxifen or tamoxifen plus genistein exhibited a marked decrease in endometrial thickness and the number of uterine glands when compared to vehicle and genistein groups (Figure 37). The hypertrophy of uterine luminal epithelium was observed in tamoxifen and tamoxifen plus genistein groups (Figure 38)

The ovarian morphology of rats treated with vehicle and genistein showed normal ongoing folliculogenesis and corpus luteum formation (Figure 39). Numerous corpora lutea were present in vehicle and genistein treated rats. Severe follicular cystic changes were observed in

tamoxifen and tamoxifen plus genistein treated rats (Figure 39), fewer corpora lutea were observed (Figure 40).

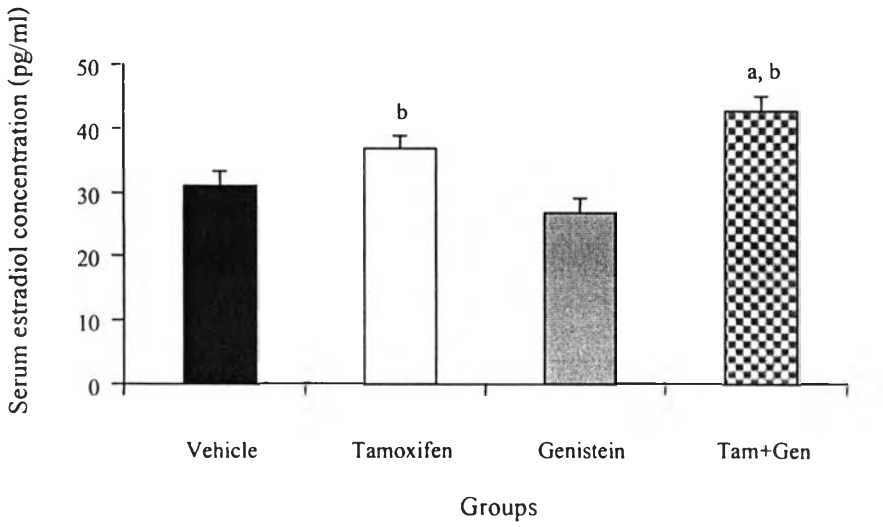

Figure 34. Serum estradiol concentration in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period.

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

Table 10. Average body weight and absolute and relative organ weights at termination

Values represent the mean ± SEM of 10 rats/ experimental group.

Treatment	Vehicle	Tamoxifen	Genistein	Tam+Gen
Body weight (g)	310.9 ± 6.2	285.4 ± 5.7[a]	298.0 ± 9.0	288.7 ± 7.3[a]
Absolute liver weight (g)	10.6 ± 0.4	8.8 ± 0.3[a,b]	10.3 ± 0.3	8.8 ± 0.4[a,b]
Relative liver weight (g/100 g BW)	3.4 ± 0.2	3.1 ± 0.1[a,b]	3.5 ± 0.1	3.2 ± 0.1[a,b]
Absolute uterine weight (mg)	413.7 ± 14.9	272.5 ± 7.3[a,b]	420.6 ± 14.9	298.3 ± 15.7[a,b]
Relative uterine weight (mg/g BW)	133.6 ± 5.6	95.5 ± 1.7[a,b]	141.3 ± 3.5	103.3 ± 4.3[a,b]
Absolute ovarian weight (mg)	130.1 ± 4.8	62.2 ± 5.1[a,b]	135.7 ± 9.5	56.6 ± 3.4[a,b]
Relative ovarian weight (mg/g BW)	42.1 ± 1.9	21.9 ± 1.8[a,b]	45.4 ± 2.6	19.6 ± 1.1[a,b]

a = significantly different from vehicle group ($p<0.05$)

b = significantly different from genistein group ($p<0.05$)

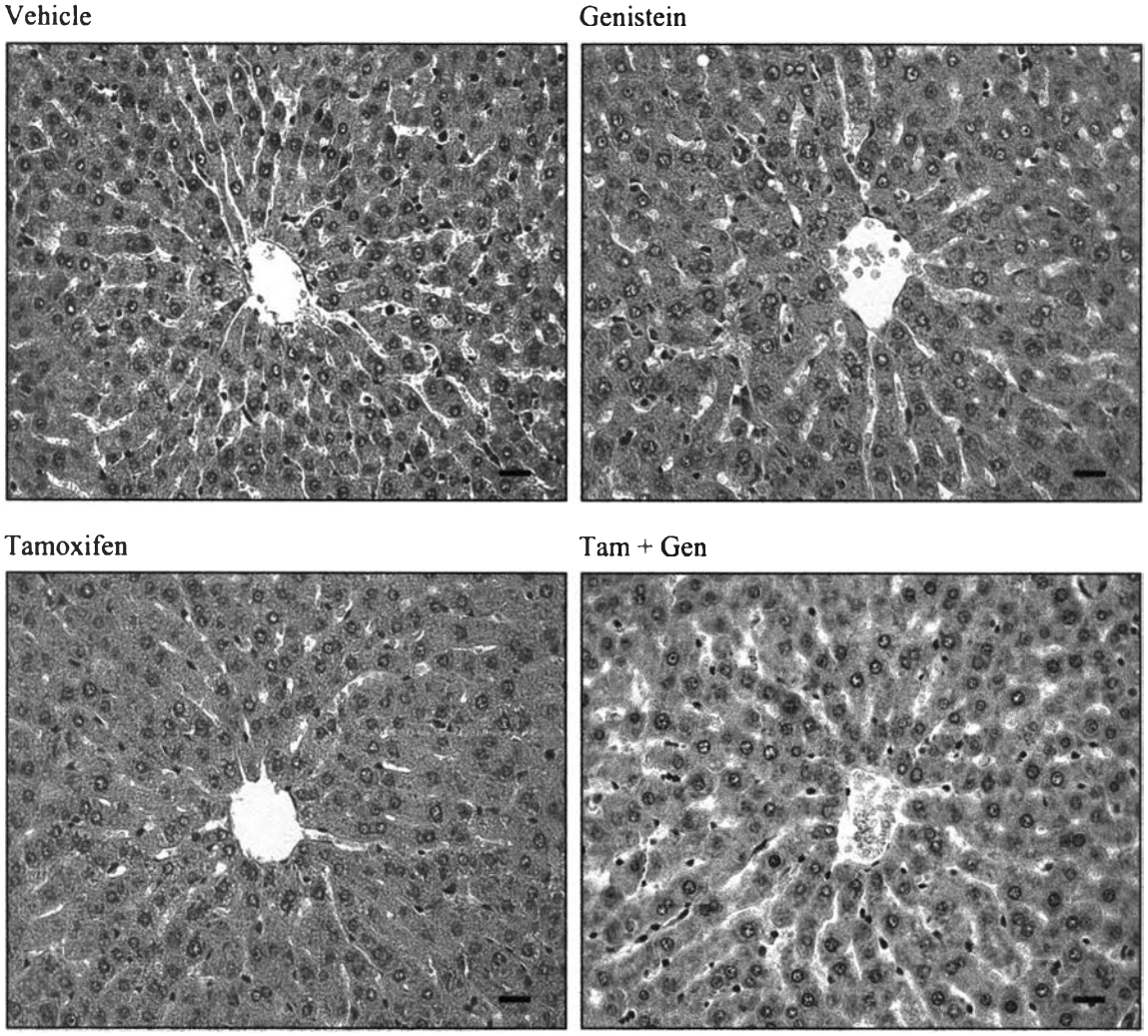

Figure 35. Comparison of histopathology alteration of liver in NMU-rats treated vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period.

H&E stain, x 200, Bar scale = 20 μM.

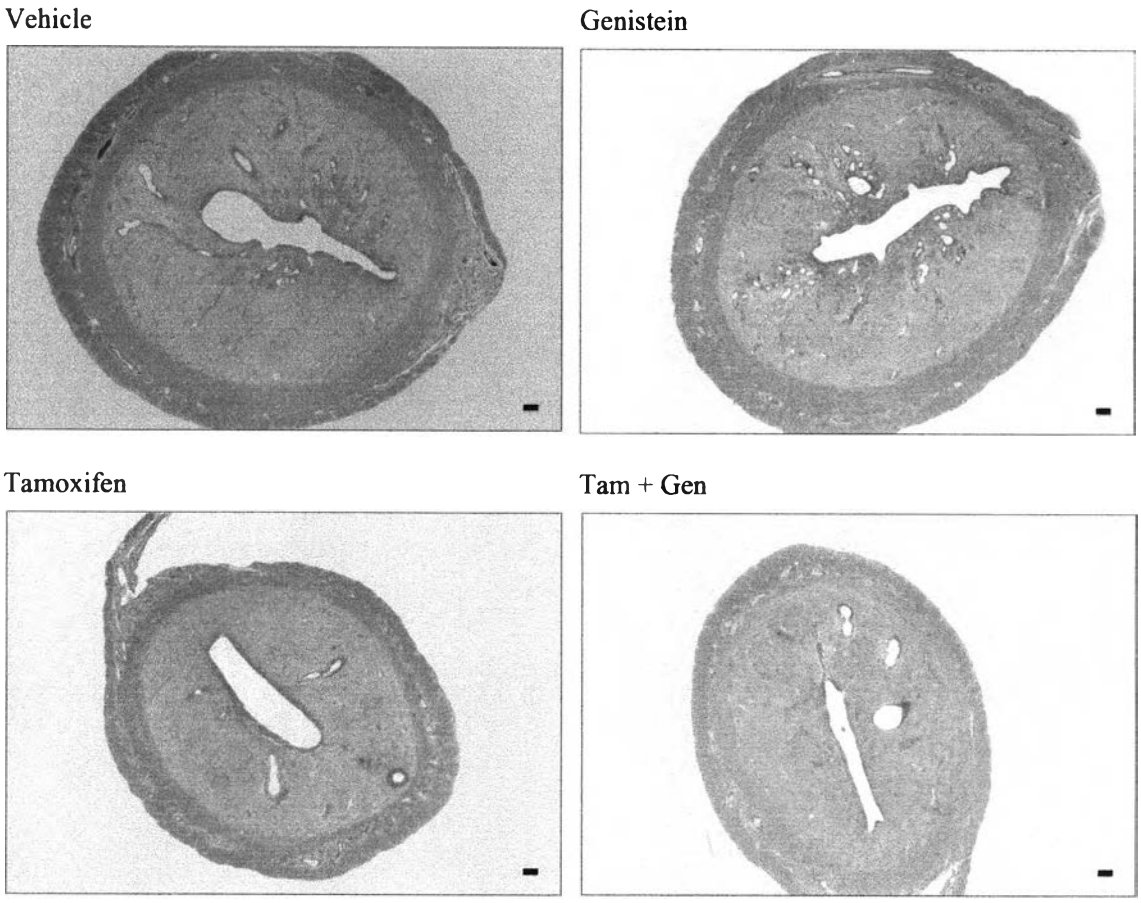

Figure 36. Comparison of histopathology alteration of uteri in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period.

H&E stain, x 20, Bar scale = 100μM.

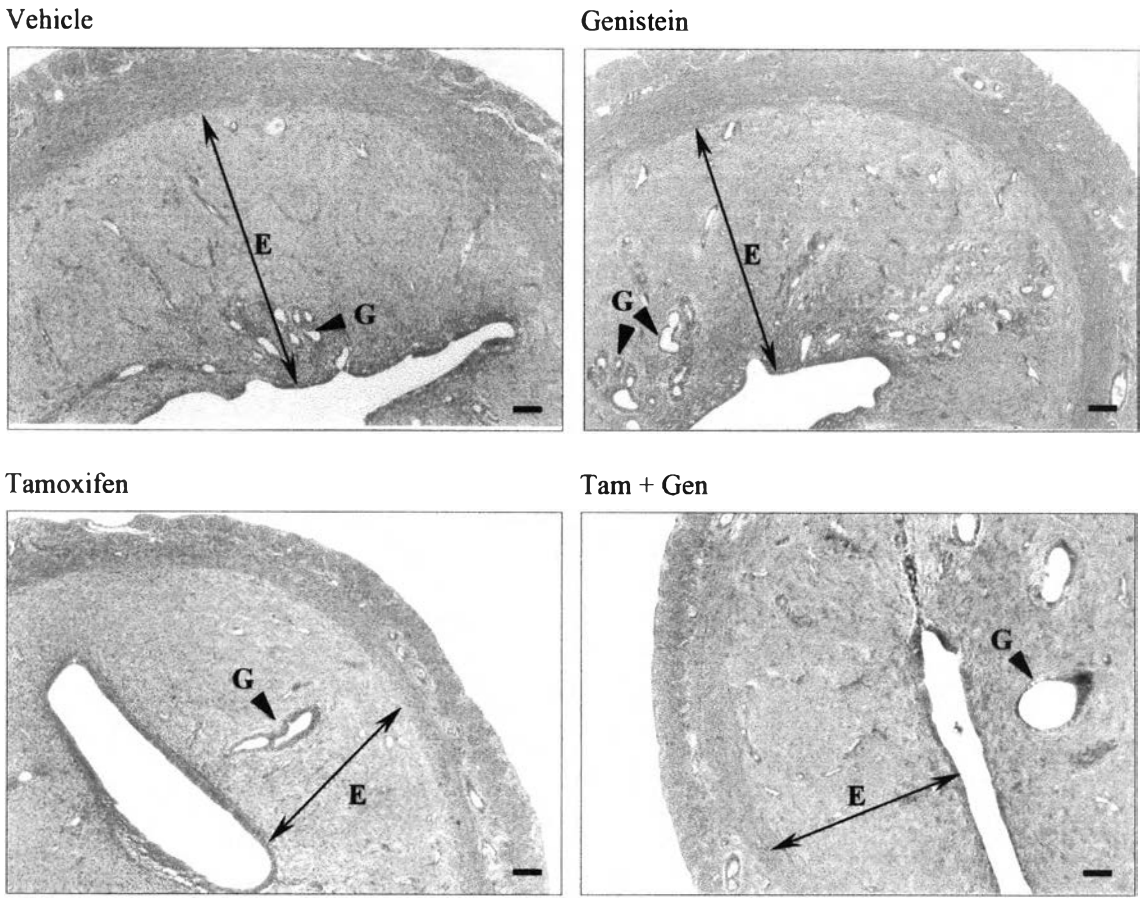

Figure 37. Comparison of histopathology alteration of uteri in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period. E = endometrium, G = uterine gland.

H&E stain, x 40, Bar scale = 100 μM.

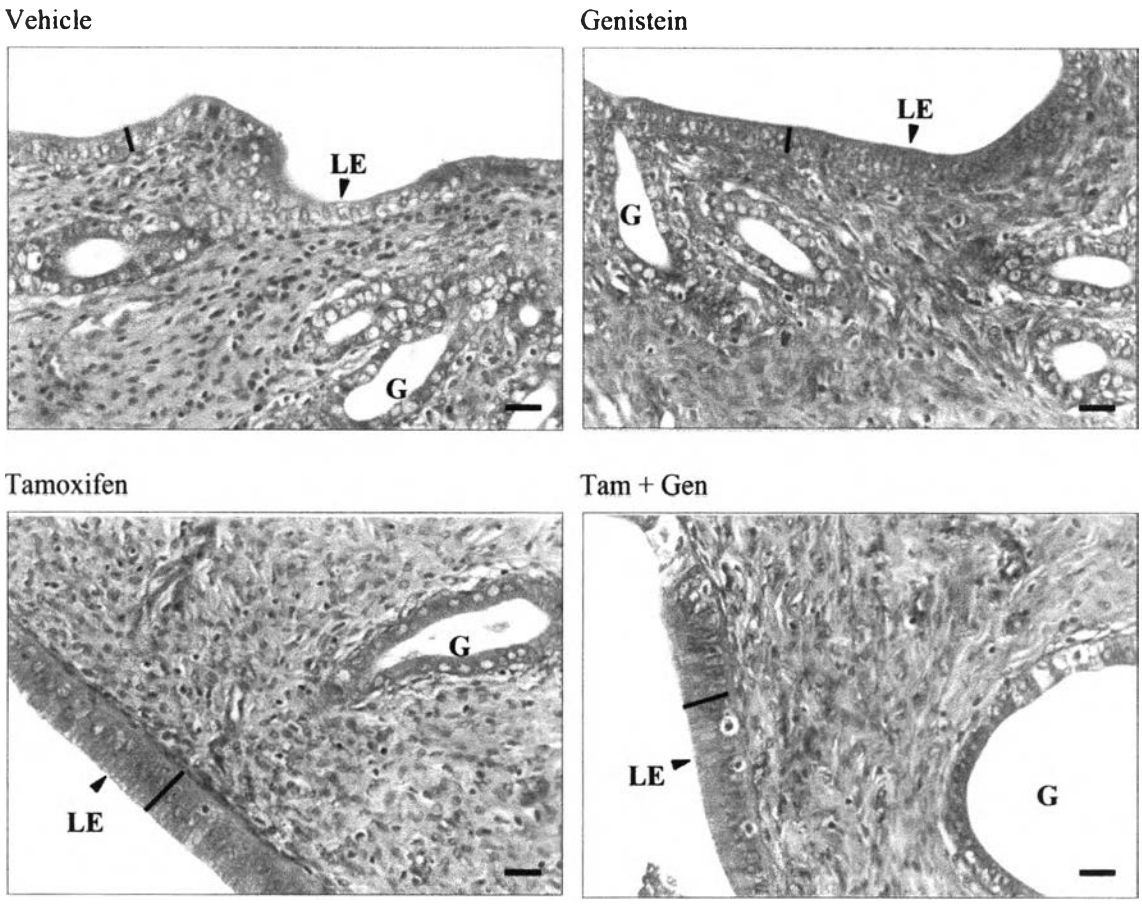

Figure 38. Comparison of histopathology alteration of uteri in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period. G = uterine gland, LE = luminal uterine epithelium.

H&E stain, x 100, Bar scale = 50 μM.

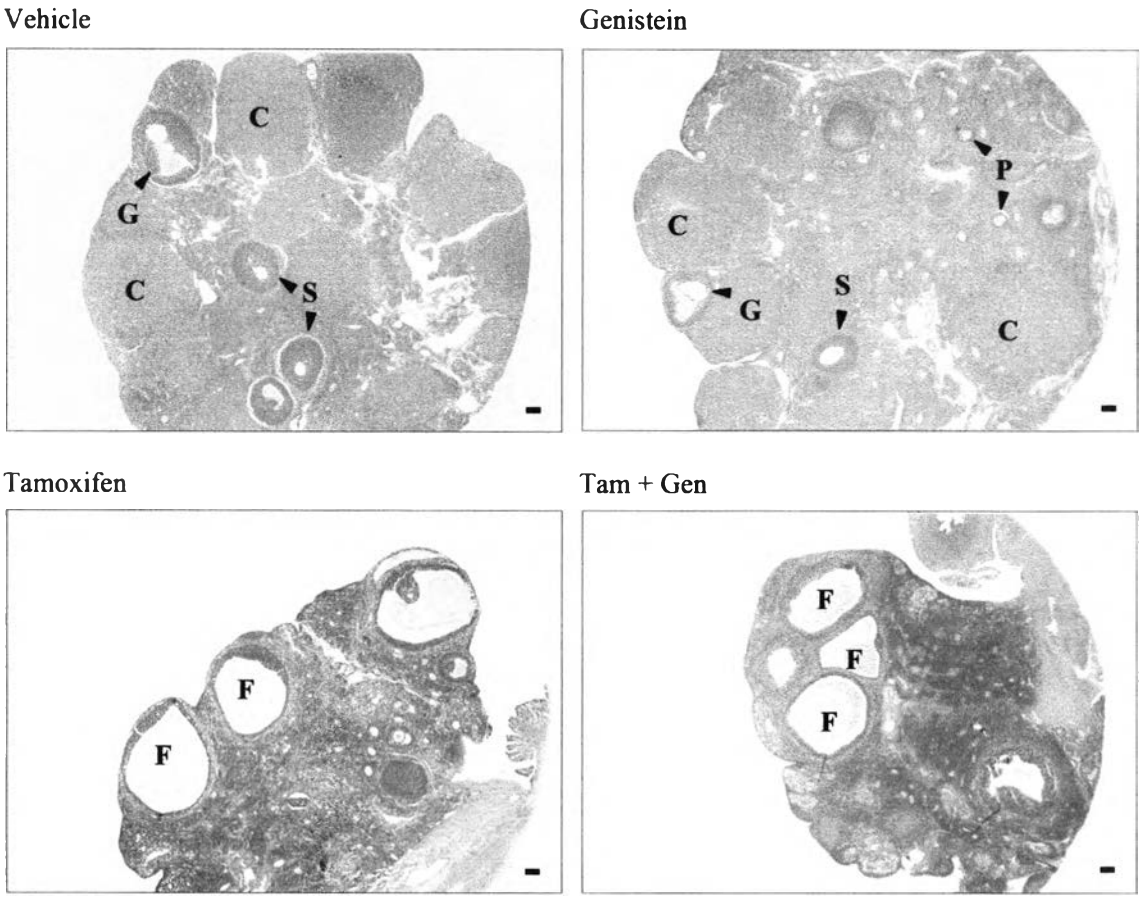

Figure 39. Comparison of histopathology alteration of ovaries in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period. P = primary follicle, S = secondary follicle, G = graafian follicle, C = corpus luteum, F = follicular cyst. H&E stain, x 20, Bar scale = 100 μM.

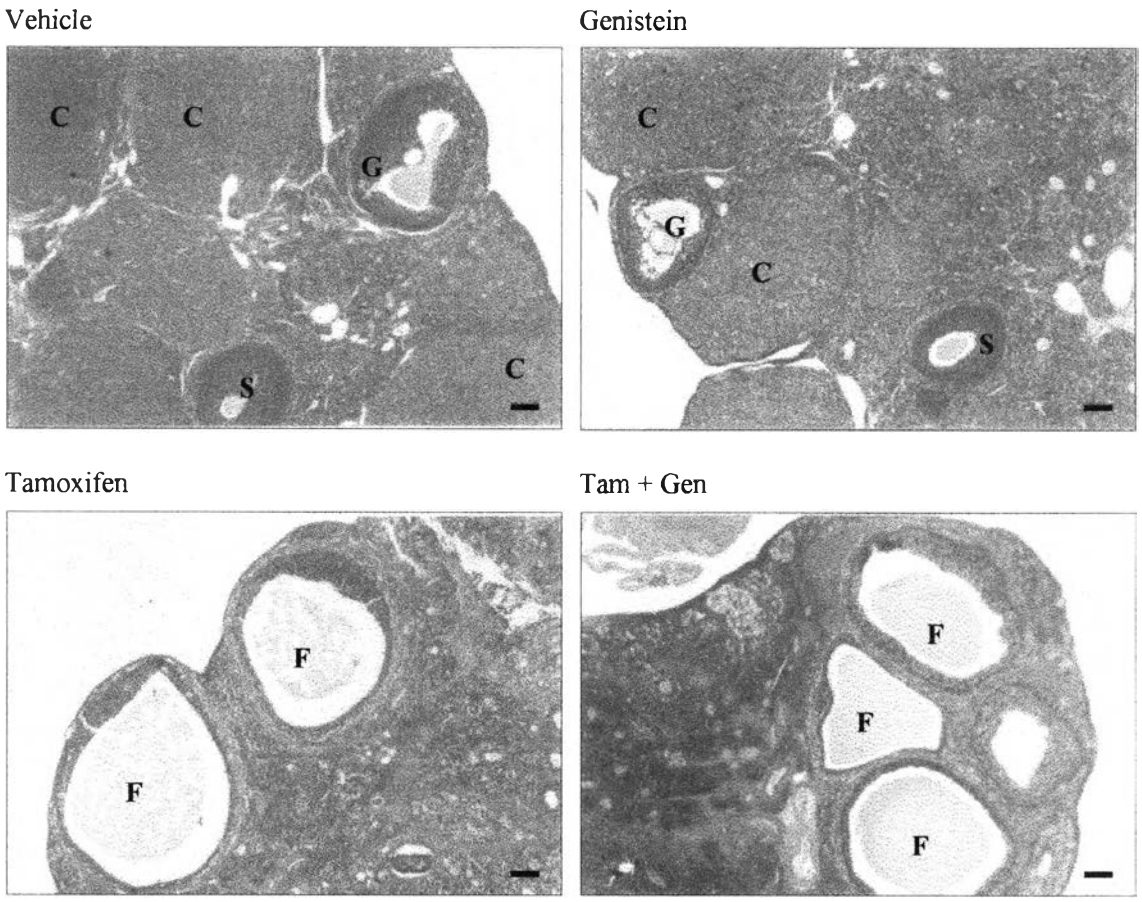

Figure 40. Comparison of histopathology alteration of ovaries in NMU-rats treated with vehicle, tamoxifen, genistein and tamoxifen plus genistein at the end of study period. S = secondary follicle, G = graafian follicle, C = corpus luteum, F = follicular cyst.

H&E stain, x 40, Bar scale = 100 μM.