CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the findings obtained from the study, which is divided into two parts. The first part was aimed to investigate the effect of three different sizes of sericins on the cell viability of colonic cell lines including cancerous cells (SW480) and normal cells (FHC). Cell cycle and cell apoptosis including certain apoptotic cellular pathways such as caspase-3 activity and Bcl-2 and Bax expression were also investigated. The second part was aimed to test the chemopreventive effect of sericin (MW 76-132 kDa) against colon tumorigenesis in rat model. The incidence of tumor and aberrant crypt foci (ACF), including the density of ACF and multiplicity of ACF in rat colons were investigated. Ki67 a cellular marker of cell proliferation and Bcl-2 an anti-apoptotic marker, colonic oxidative stress, and immunoregulatory effect were investigated to determine the potential mechanism of action of sericin against colon cancer.

Chemopreventive effect of sericin in cell culture model

1. Effect of sericin on viability of SW480 cells

In the present study, we investigated the effect of three types of sericin, sericin A (MW 191-339 kDa), B (MW 76-132 kDa) and C (MW 61-113 kDa) on SW480 cancerous colonic cell viability using MTT assay. This method is based on the quantification of purple-colored formazan, which is formed by the reduction of MTT. The degree of formazan production refers to the number of active mitochondria in the living cells. The PBS-treated cells were used as a negative control for the comparison of cell viability. Human colon cancer SW480 cells were treated with different concentrations (25, 50, 100, 200, 400, 800 and 1,600 μg/ml) of each type of sericin for 24, 48 or 72 h. The results showed that most concentrations of all of sericin did not obviously affect the viability of SW480 cells after 24 h of incubation (Figure 5). Although sericin C at 400 μg/ml significantly decreased SW480 cell viability when compared to PBS-treated cells, this treatment showed cell viability more than 80%.

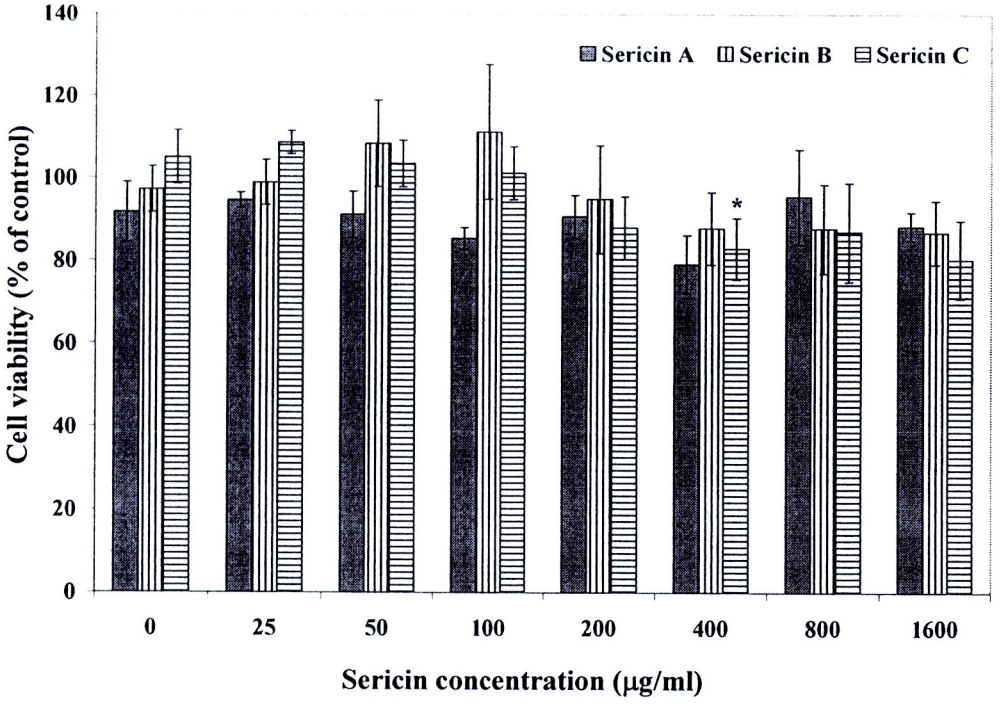


Figure 5 Viability of SW480 cells treated with various concentrations of sericin for 24 h.

Note: SW480 cells were treated with different concentrations of sericin A, B and C for 24 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. $^{*}p \leq 0.05$, compared to control cells (un-treated cells).

For longer incubation period as shown in Figure 6, treatment of SW480 cells with sericin B (100-1,600 μg/ml) and sericin C (50-1,600 μg/ml) for 48 h significantly decreased SW480 cell viability in a dose-dependent manner. Sericin A did not significantly affect cell viability of SW480 cells at any concentrations. Although statistically significant reduction was observed, the highest reduction of cells viability in this experiment was approximately 30%.

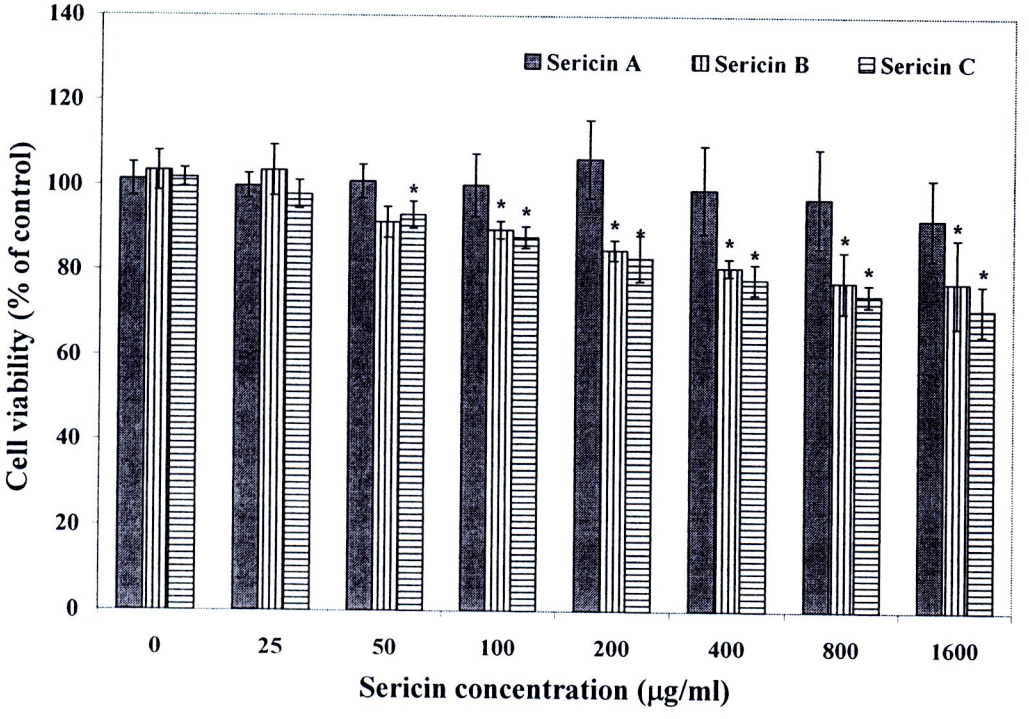


Figure 6 Viability of SW480 cells treated with various concentrations of sericin for 48 h.

Note: SW480 cells were treated with different concentrations of sericin A, B and C for 48 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. * $p \leq 0.05$, compared to control cells (un-treated cells).

When SW480 cells were incubated with sericin for 72 h, most sericin at higher concentrations (≥ 400 μg/ml) significantly reduced the viability of these cells (Figure 7). The decrease in cell viability was dependent on sericin concentration. Similar to 24 and 48 h incubation periods, 72 h treatment of sericin B and C showed higher inhibitory effect on the viability of SW480 cells than that of sericin A. The highest reduction of cell viability was approximately 40% by sericin B and C at 1,600 μg/ml. Among three types of sericin, smaller size sericin (sericin B and C) seems to be more effective to reduce SW480 cell viability than large size sericin (sericin A).

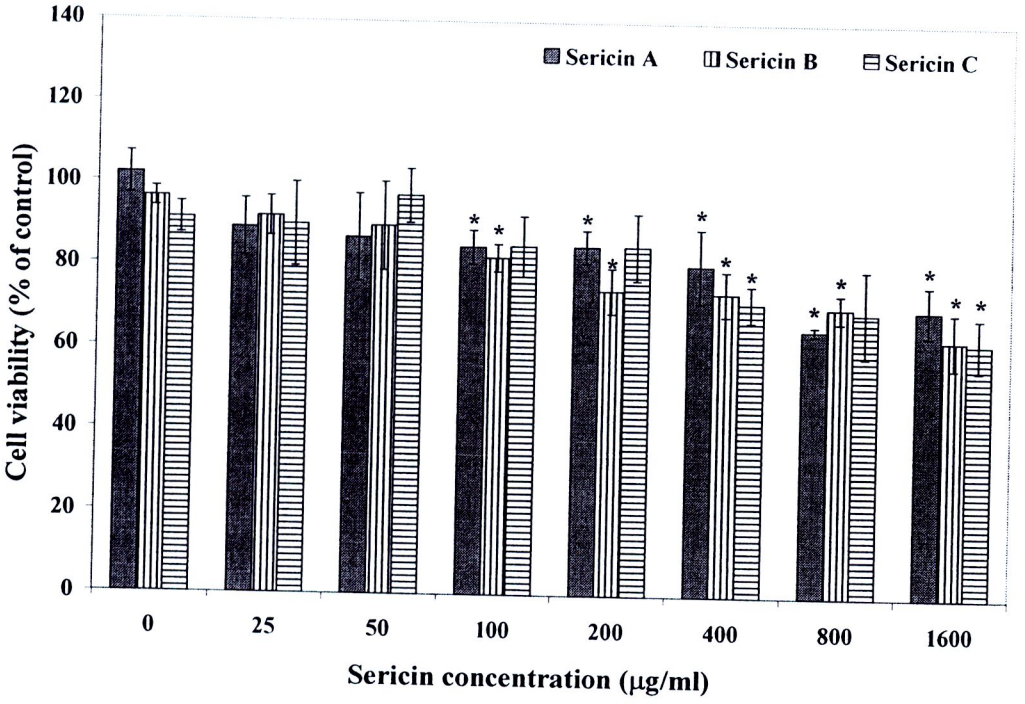


Figure 7 Viability of SW480 cells treated with various concentrations of sericin for 72 h.

Note: SW480 cells were treated with different concentrations of sericin A, B and C for 72 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. * $p \leq 0.05$, compared to control cells (un-treated cells).

2. Effect of sericin on viability of FHC cells

Since sericin was shown to reduce the viability of cancerous colonic SW480 cells, sericin was then tested whether it would have the same effect on normal colon cells. Therefore, the effect of sericin on the cell viability of FHC normal colon cells was examined at the same concentrations and treatment period as used for SW480 cells. As shown in Figure 8, the viability of FHC cells at 24 h was minimally affected by all types of sericin treatment, although during 100-400 μg/ml of sericin C decreased FHC cell viability. For 24 h period, the percentages of FHC cell viability were still higher than 80% after most sericin treatments.

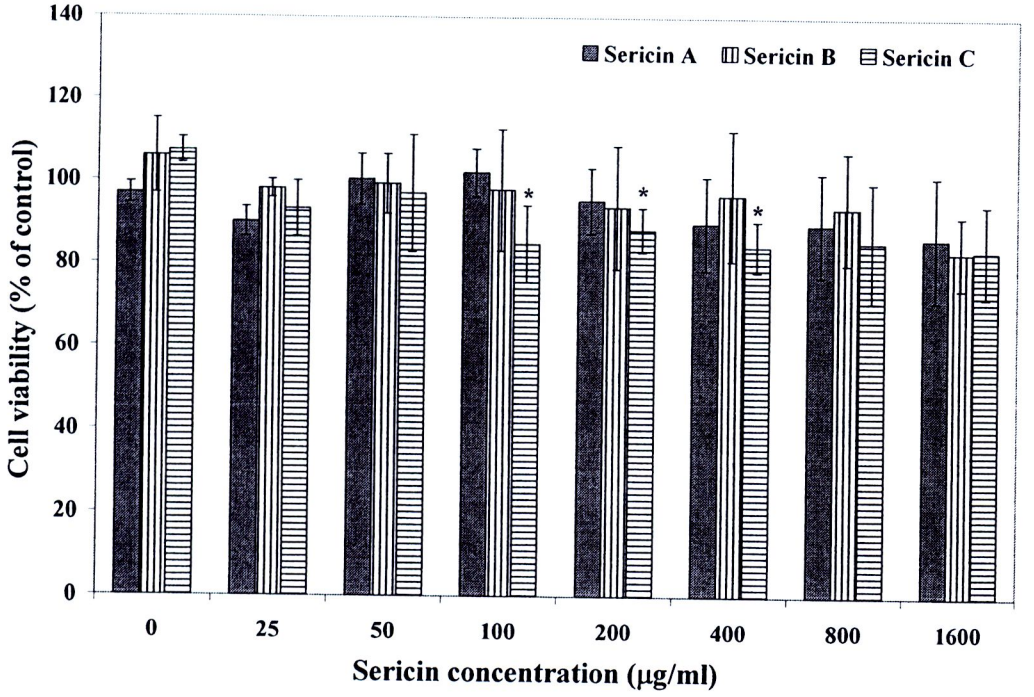


Figure 8 Viability of FHC cells treated with various concentrations of sericin for 24 h.

Note: FHC cells were treated with different concentrations of sericin A, and C for 24 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. * $p \leq 0.05$, compared to control cells (un-treated cells).

After treatment for 48 h, all types of sericin slightly but significantly decreased FHC cell viability in a dose-dependent manner (Figure 9). Similarly to 48 h treatment, the viability of FHC cells was gradually decreased with increasing concentration of sericin after 72 h of treatment (Figure 10). Taken all data together, all three types of sericin have small effect on cell viability of both SW480 and FHC cells since cell viabilities were reduced only 30-40% at highest of tested concentration (1,600 μg/ml). These findings suggest that sericin may be considered as a low cytotoxic agent for both cancer and normal colon cells.

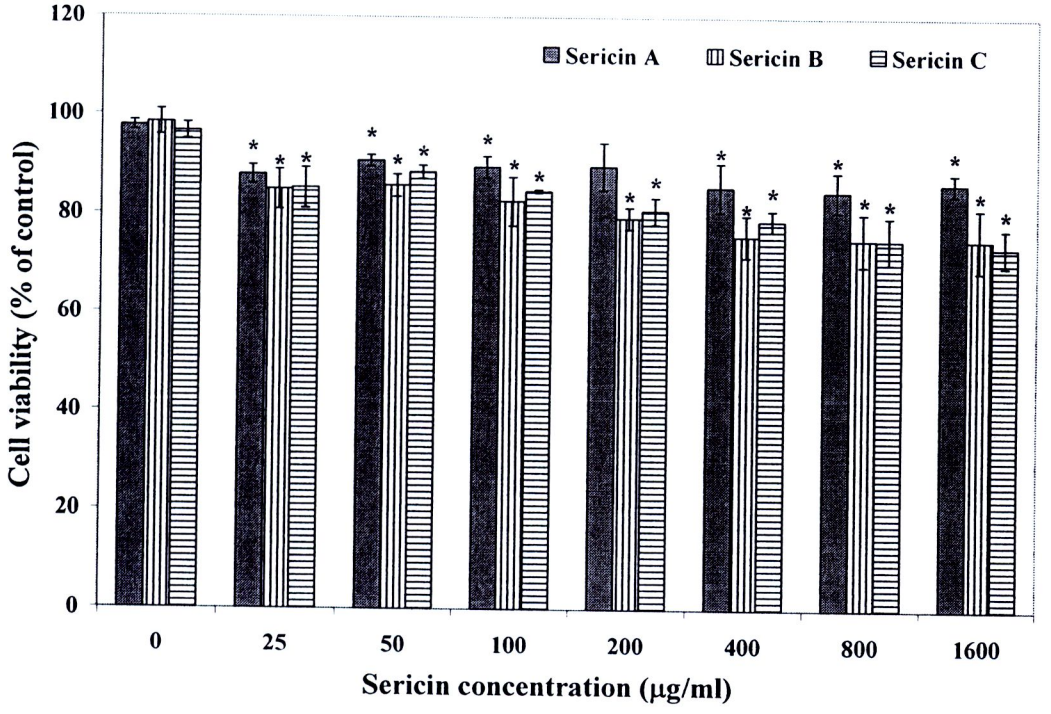


Figure 9 Viability of FHC cells treated with various concentrations of sericin for 48 h.

Note: FHC cells were treated with different concentrations of sericin A, B and C for 48 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. * $p \leq 0.05$, compared to control cells (un-treated cells).

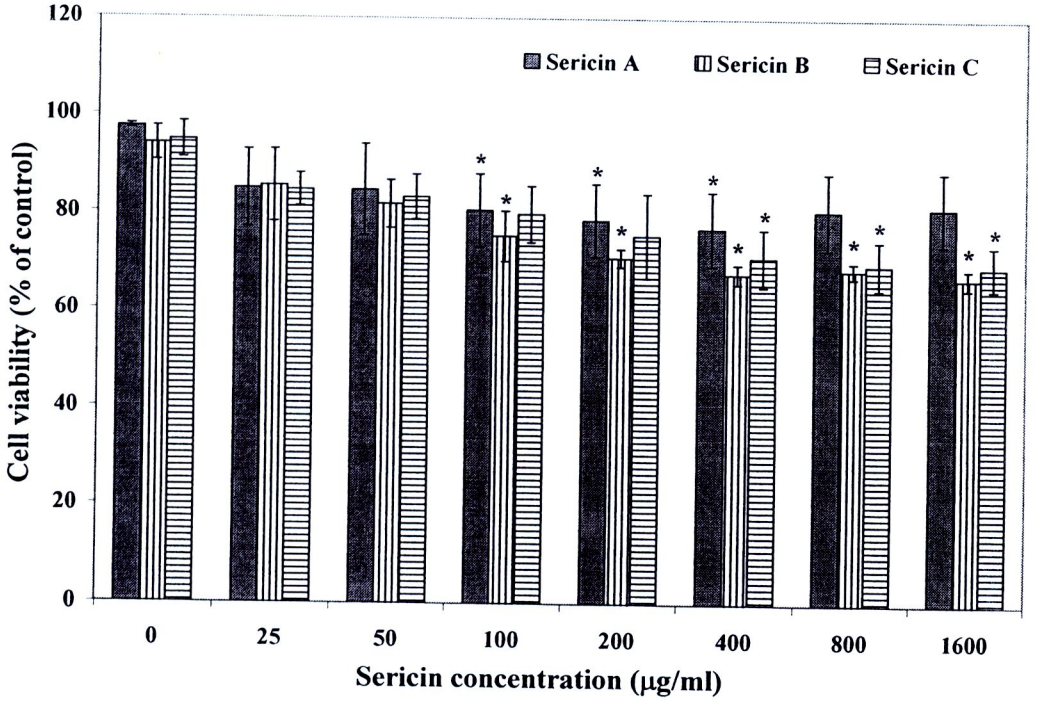


Figure 10 Viability of FHC cells treated with various concentrations of sericin for 72 h.

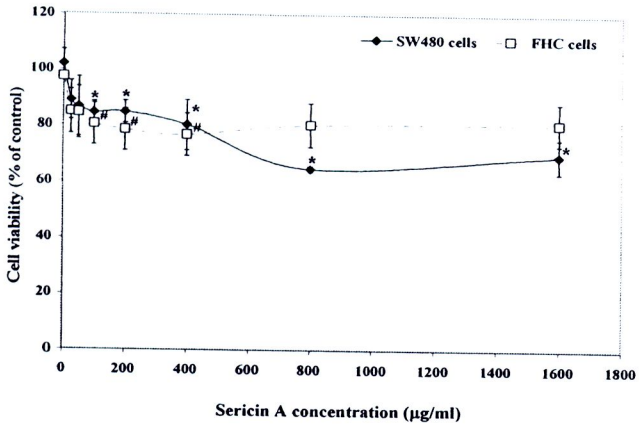
Note: FHC cells were treated with different concentrations of sericin A, B and C for 72 h, and their viability was determined by MTT assay. The cell viability was calculated as percentage of viable cells in treated condition relative to un-treated condition. The values are means ± SEM of four experiments. * $p \leq 0.05$, compared to control cells (un-treated cells).

3. Comparison the effect of sericin on SW480 and FHC cell viability

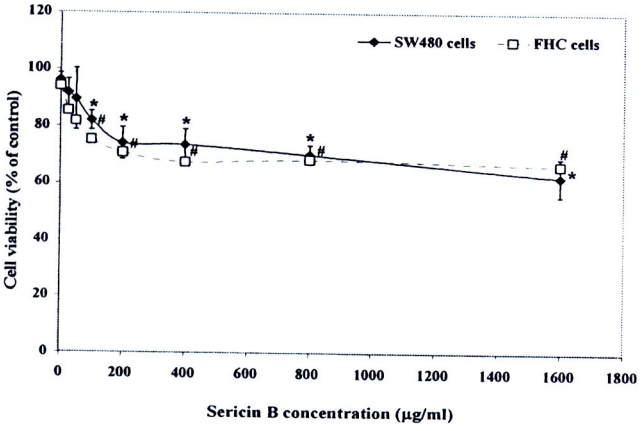
In addition to damaging cancer cells, many chemopreventive agents can also damage or induce cell death of normal cells. An ideal chemopreventive agent should have minimal or no toxicity to normal cells or tissue. Figure 11 shows the effect of sericin on viability of cancerous SW480 in comparison with normal FHC cells. Large sericin (sericin A) seemed to reduce the viability of colonic adenocarcinoma SW480 better than normal FHC cells (Figure 11A), whereas small sericin (sericin B and C) did not show such differences (Figure 11B and 11C). These results suggest that sericin possessed either a moderate inhibitory effect to cell growth or proliferating effect for colonic cells. According to the incubation of sericin at various concentrations for 72 h, all types of sericin at 1,600 μg/ml showed a significant reduction of SW480 and FHC cell viability (Figure 11). Therefore, we used this condition to test the mechanisms of sericin that affect the reduction of cell viability.

Sericin used in this study was prepared in a specific technique of isolation that provided sericin with three ranges of molecular weight, sericin A (191-339 kDa), sericin B (76-132 kDa) and C (61-113 kda). From the literature, different size sericin is thought to differently exhibit some biological properties. Biological activities of sericin are associated with its molecular weight. Small size sericin (5-100 kDa) rather than large size sericin (50-200 kDa) can accelerate the proliferation of hybridoma cells [11]. The present results showed that large size sericin (191-339 kDa) and small size seicin (61-132 kDa) significantly reduced SW480 and FHC cell viability, but the small size showed higher inhibition. The results suggest that the chemopreventive effect of sericin is associated with its size. However, the reason for this difference between small and large size sericin is not clear at this time.

A. Sericin A



B. Sericin B



C. Sericin C

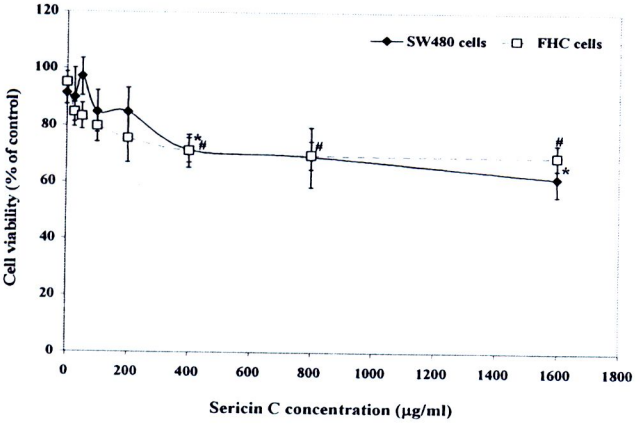


Figure 11 Viability of SW480 and FHC cell treated with various concentrations of sericin for 72 h.

Note: SW480 and FHC cells were treated with different concentrations of sericin A (A), sericin B (B) and sericin C (C) for 72 h, and their viability was determined by MTT assay. The values are means ± SEM of four experiments. * or # $p \leq 0.05$, compared to control cells (un-treated cells).

4. Effect of sericin on SW480 and FHC cell apoptosis

Sericin at 1,600 μg/ml and 72 h treatment showed the significant reduction of SW480 and FHC cell viability, we then examined whether these cells undergo cell apoptosis or necrosis. Double staining method using FITC-conjugated annexin V and propidium iodide (PI) by flow cytometer analysis was conducted. Annexin V binds preferentially to a negatively charged inner membrane phosphatidylserine (PS) exposed to the cell surface of apoptotic cells. The impermeable vital dye PI enters and binds to DNA of cells undergoing necrotic changes with damaged plasma membrane. In this particular experiment, SW480 cells were treated with each type of sericin at 1,600 μg/ml for 72 h. The results showed that only sericin B and C, but not sericin A significantly increased the percentage of late apoptotic cells (annexin and PI-positive cells) as shown in Figure 12A. All three sericins did not change the percentage of necrotic cells (PI-positive cells) compared to PBS-treated cells. For FHC normal colon cells, there were no changes in the percentage of both apoptotic and necrotic cells after treatment with all types of sericin compared to control and PBS-treated cells (Figure 12B). These results suggest that cancerous SW480 and normal FHC colon cells differently respond to sericin treatment.

The chemopreventive of candidate compounds can be demonstrated by testing their ability to suppress cancer cell proliferation, induce cell differentiation, inhibition angiogenesis, and induce cell cycle arrest. Among these effects, cell apoptosis is the major targets for the treatment and prevention of colon cancer [79, 139]. The present results indicate that the sericin induces apoptosis of SW480 colon cancer cells, but not of FHC cells. These observations indicate that sericin may potentially be useful in the prevention of colon carcinogenesis.

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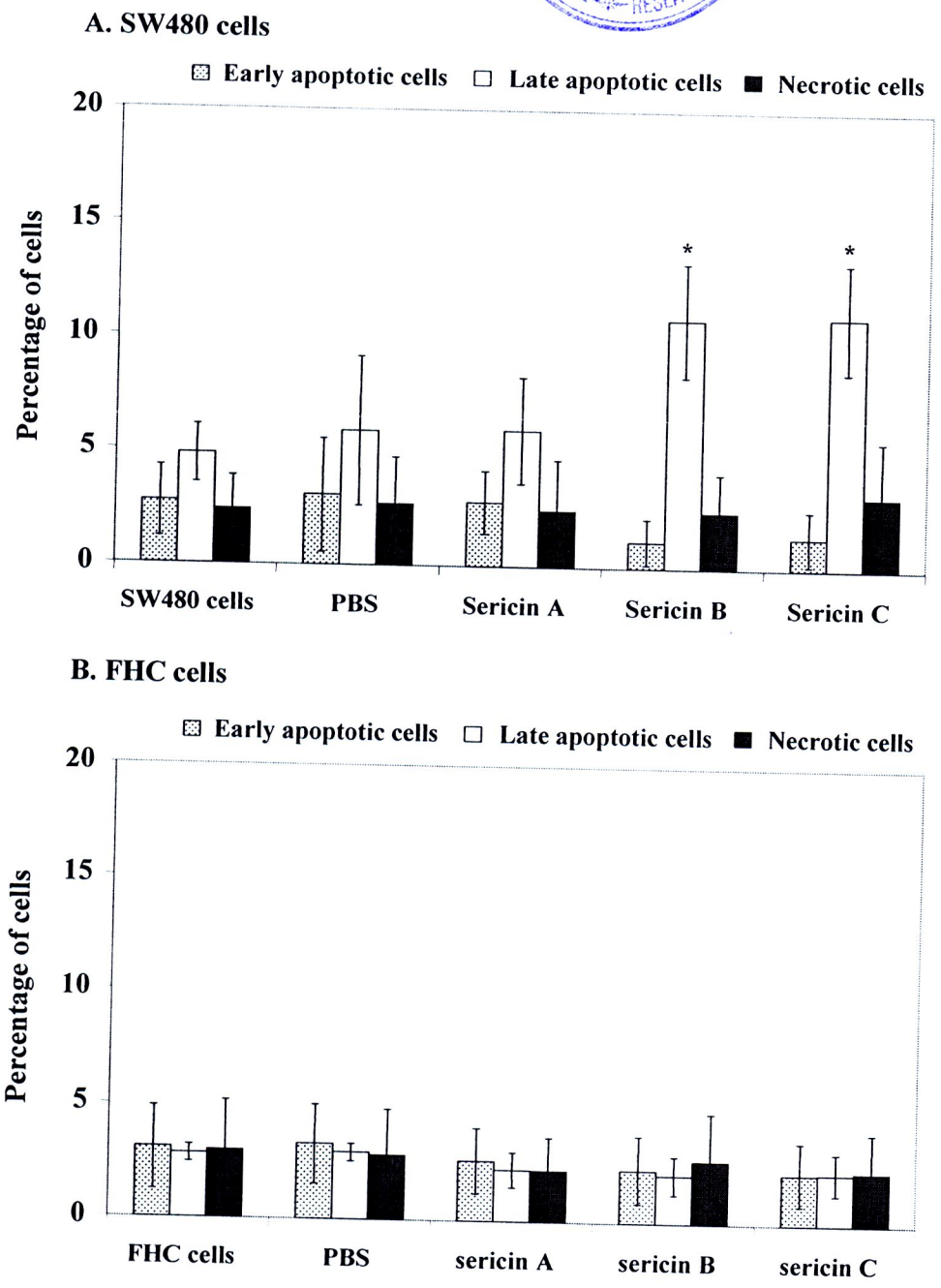

Figure 12 Effect of sericin on apoptosis of SW480 (A) and FHC (B) cells.

Note: SW480 and FHC cells were treated with sericins at 1,600 μg/ml for 72 h. Apoptotic cells were determined by flow cytometric analysis. The values are the means ± SEM of four experiments. * $p \leq 0.05$, compared to control (un-treated cells).

5. Effect of sericin on caspase-3 activity in SW480 cells

Apoptosis requires the activation of caspases, so the involvement of caspase activation was investigated to confirm sericin-induced apoptosis in SW480 colon cancer cells. Caspases, also known as cysteine aspartate-specific proteases, are a family of intracellular proteins involved in the initiation and execution of apoptosis [140]. Initiator caspases (caspase-2, -8, -9, 12) are able to activate effector caspases (caspase-3, -6, -7) or amplify the caspase cascade [141]. Thus caspases are important in the execution of apoptotic cells, at least in some apoptosis signal transduction. In this experiment, the activity of caspase-3 was examined using fluorogenic caspase-3 substrate, Ac-DEVD-AMC. SW480 colon cancer cells were treated with sericin at a concentration of 1,600 μg/ml for 72 h, and whole cell lysates were collected for analysis. As shown in Figure 13, SW480 cells treated with sericin at 1,600 μg/ml for 72 h exhibited the increase caspase-3 activity activities. Only sericin B significantly enhanced caspase-3 activity in SW480 cells.

The mechanisms of apoptosis mainly involve two signaling pathways, namely the intrinsic pathway, involving the activation of the mitochondria and several caspases and their extrinsic pathway, involving the activation of death receptors [85]. The key element in mitochondrial pathway is the release of cytochrome C from mitochondria to cytosol, binds to Apaf-1 and caspase-9 and the latest activation of caspase-3 [142]. We demonstrated that sericin induced cell apoptosis in SW480 cells via activation of caspase-3. Caspase-3 is one of executioner caspases which its activity is increased when the cells decide to undergo apoptosis [140, 141]. The data suggest that sericin induces adenocarcinoma colonic cells to undergo apoptosis through caspase-3 dependent pathway.

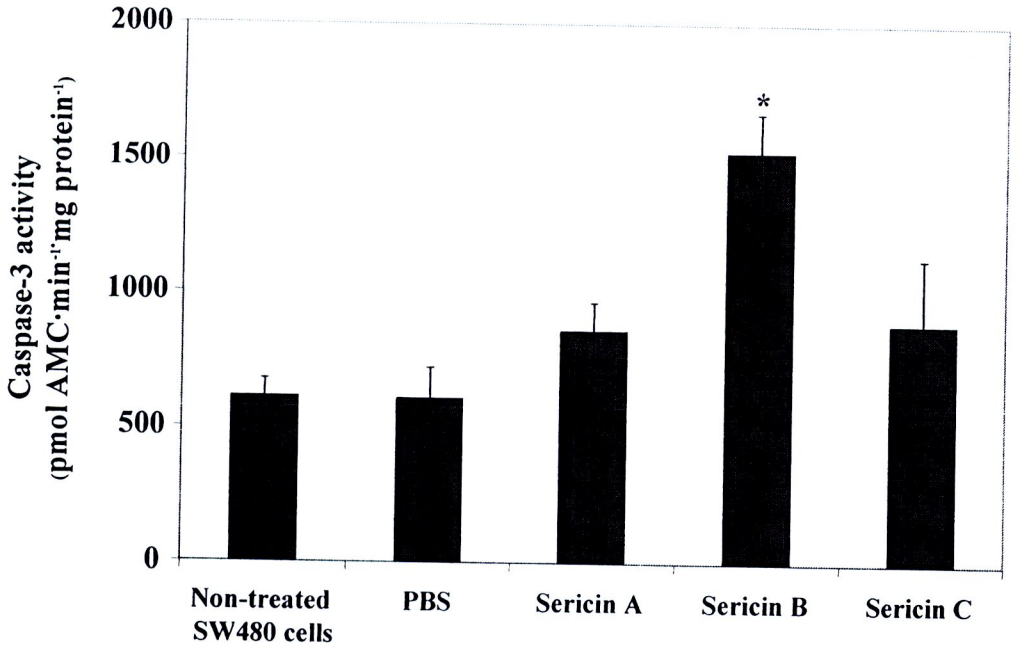

Figure 13 Effect of sericin on caspase-3 activation of SW480 after incubation for 72 h.

Note: SW480 cells were treated with three sericins at a concentration 1,600 μg/ml for 72 h. Caspase-3 activation was determined by Caspase-3 fluorimetric kit. The values are the means ± SEM of four experiments. * $p \leq 0.05$, compared to control (un-treated cells).

6. Effect of sericin on expression of Bcl-2 and Bax

The expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax are also expected to be correlated to the degree of apoptosis of SW480 cells in previous experiment. The expressions of these proteins were examined by using western blotting. SW480 cells were treated with three types of sericin at the same condition as mentioned earlier. As shown in Figure 10, there was a slight reduction of Bcl-2 protein level in SW480 cells treated with sericin for 72 h, but only sericin C showed a significant reduction. The expression of Bax protein in sericin-treated cells did not differ from that in the control cells (Figure 14).

Apoptosis regulatory proteins associated with the mitochondrial pathway is controlled by Bcl-2 family proteins [143, 144]. The Bcl-2 is the protein families that have to regulate cell apoptosis either to induce or inhibit apoptotic process. Bcl-2 is anti-apoptotic whereas Bax is pro-apoptotic protein [145, 146]. Bcl-2 was down-regulated but the pro-apoptotic protein Bax remained unchanged. This result suggests that the pro-apoptotic effects of sericin, especially sericin C may be mediated by its ability to lower Bcl-2 protein levels, which may then enhance colon cancer SW480 cells to undergo an apoptotic process. However, the reduction of FHC cell viability by sericin was not associated with sericin-induced cell apoptosis. The results suggest that sericin might decrease cell viability by other mechanism such as cell cycle arrest.

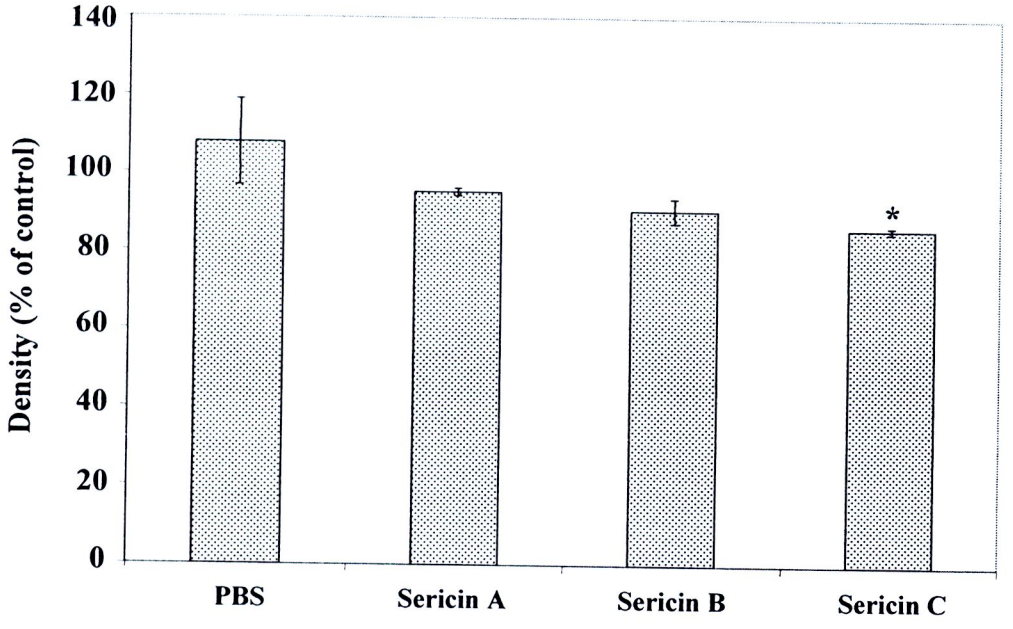

Figure 14 Effect of sericin on Bcl-2 protein expression of SW480 cells after incubation for 72 h.

Note: SW480 cells were treated with three sericins at a concentration 1,600 μg/ml for 72 h. Bcl-2 protein expression was determined by Western Blotting. The values are the means ± SEM of four experiments. * $p \leq 0.05$, compared to control (un-treated cells).

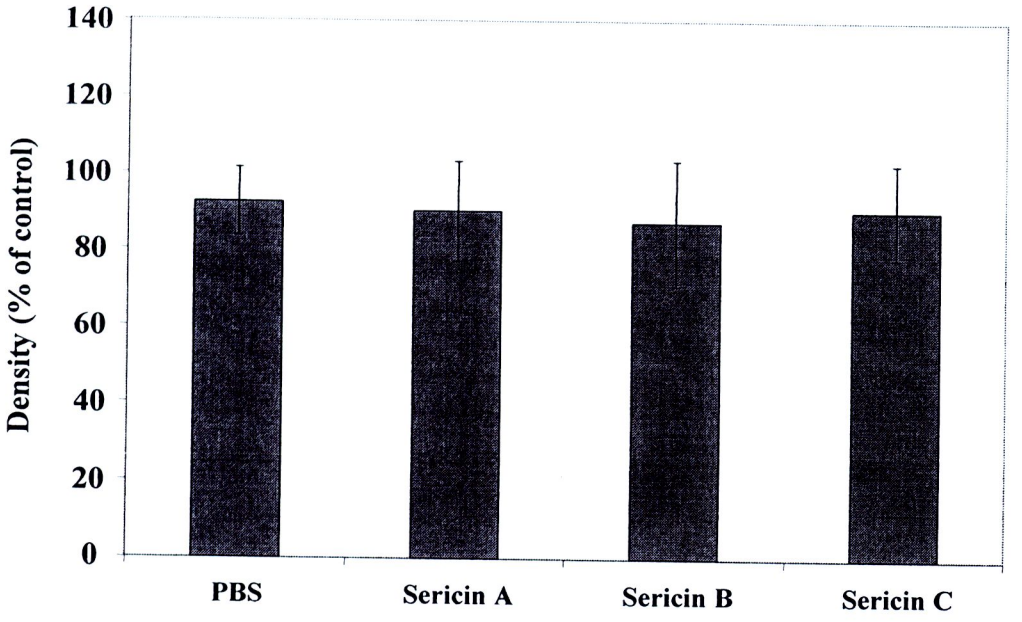

Figure 15 Effect of sericin on Bax expression of SW480 after incubation for 72 h.

Note: SW480 cells were treated with three sericins at a concentration 1,600 μg/ml for 72 h. Bax protein expression was determined by Western Blotting. The values are the means ± SEM of four experiments.

7. Effect of sericin on cell cycle

To further investigate whether or not the inhibitory effect of sericin on SW480 cell viability might partially be due to an arresting of cell proliferation, a cell cycle analysis was also conducted by using flow cytometry. After treating the SW480 cells with sericin at 1,600 for 72 h, they were harvested and stained with PI. The distribution of cells in different phases of the cycle is illustrated in (Table 5). When compare between carcinoma and normal colonic cells, the colon cancer SW480 cells had a higher percentage of cells in the S phase (19.22 ± 6.33%) than FHC normal colonic cells (7.64 ± 1.01%). More population in the S phase of SW480 cells may imply that carcinoma cells have a higher degree of DNA synthesis than that of normal FHC cells. After treatment with all types of sericin, the percentage of cells in the G0/G1 phase were increased and the percentage of cells in S phase of SW480 cells were decreased from the control level although those reductions were not statistically significant.

Cell cycle is a complex process involved in the growth of proliferating of cells. The defect in normal pattern of cell cycle leads to excessive proliferation and finally results in the formation of cancerous cells [96]. To investigate whether sericin also regulates the cell cycle progression, the present study determined its effect on cell cycle distribution by flow cytometry analysis. The results found that sercin had no effects on cell cycle of SW480 cells, consistent with the study of anti-cancer drug, oxaliplatin, showed no change the pattern of cell cycle of SW480 cells differ from control cells [147]. Sericin treatment tended to increase percentage of SW480 cells in G0/G1 phase. For instance, caffeic acid phenyl ester (CAPE) have been shown to inhibit SW480 cell proliferation by inducing cell cycle arrest at G0/G1 phase [148]. These results suggest that the inhibitory effect of sericin on colon cancer SW480 cell viability be partially due to the disruption of cell cycle.

Interestingly, the results have shown that cell cycle seems to be accumulated in S phase but less G2/M phase in FHC normal colon cells. This is why, the FHC cell viability is decreased in sericin treatment by preventing the cells transit from S to G2/M phase. Moreover, the cell cycle is accumulated in S phase of FHC normal cells, could be due to stimulation effect of sericin on cell proliferation. This finding agrees with Terada et al. reported that sericin supplement could accelerates the proliferation

of several mammalian cell lines [135]. These results suggest that the cell cycle of adenocarcinoma and normal colonic cell lines be differently regulated by sericin treatment. Sericin seems to have paradoxical effect, it may inhibit cell proliferation in the rapid-proliferative cells (cancerous cells) and it could stimulate cell proliferation in slow-proliferative cells (normal cells).

Table 5 Effect of various concentrations of sericin on SW480 cell cycle

Treatment	SW480 cells			FHC cells		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Untreated	60.46 ± 6.17	19.22 ± 6.33	16.49 ± 8.09	69.79 ± 4.04	7.64 ± 1.01	17.08 ± 3.02
PBS	66.20 ± 2.67	13.54 ± 3.08	20.11 ± 5.90	68.56 ± 5.12	7.90 ± 2.60	17.06 ± 3.72
Sericin A	67.91 ± 1.41	11.00 ± 2.13	20.21 ± 0.66	67.23 ± 8.01	10.02 ± 1.57	15.02 ± 2.89
Sericin B	72.37 ± 5.58	11.11 ± 2.61	17.01 ± 5.09	68.43 ± 8.10	10.24 ± 2.90	13.57 ± 1.32
Sericin C	68.65 ± 1.67	9.81 ± 4.57	18.95 ± 1.30	71.51 ± 4.80	9.61 ± 0.37*	12.65 ± 4.36

Note: Values are mean ± SD

* $p \leq 0.05$, compared to control un-treated cells.

Chemopreventive effect of sericin in animal model

1. Effect of sericin on body weight and food consumption

Table 6 presents the initial, final body weights and body weight gain of control rats and DMH-treated rats. No statistically significant difference was observed in body weights and body weight gain among all groups of rats. Body weight gain in DMH-treated rats was not different from normal rats. This might suggest that tumorigenesis development in DMH-treated rats was in the early stage so that the body weight was not yet reduced.

As shown in Table 7, food consumption of control casein and sericin diet was 10 -12 g/day/rat, which was not different from that of DMH-treated groups in the initiation period (first week). Food consumption of sericin-fed rats and receiving DMH was significant lower than that of control sericin group. At the final period (20^{th} weeks), all DMH-treated groups (group 3-5) had slightly higher food consumptions compared to control normal rats (casein-fed rats). This result suggests that rats having colon tumorigenesis consume more food than normal rats. It is possible that colon tumorigenesis may effect on the absorption of certain nutrients, thus DMH-treated rats would require higher amount of food. However, the exact reason for this phenomenon is unknown. These results also showed that type of protein; casein or sericin, has no different effect on body weight or food consumption. In addition, by visual observation, the animals appeared to be in good health during the entire experimental period (Table 8).

Table 6 Effect of sericin on initial, final body weight and body weight gain

Treatment	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
G1 Casein	237.38 ± 11.61	502.75 ± 28.25	265.37 ± 16.64
G2 Sericin	234.67 ± 30.53	488.83 ± 40.81	254.16 ± 10.28
G3 Casein + DMH	228.54 ± 37.24	498.08 ± 29.73	269.54 ± 7.54
G4 Sericin + DMH	206.04 ± 25.24	483.38 ± 24.84	277.34 ± 0.40
G5 Post-sericin + DMH	229.00 ± 35.06	486.83 ± 48.42	257.83 ± 13.36

Note: Values are mean ± SD of 6 rats.

Table 7 Initial and final food consumption

Treatment	Initial food consumption (g/rat/day)[a]	Final food consumption (g/rat/day)[b]
G1 Casein	10.73 ± 2.76	18.21 ± 0.90
G2 Sericin	12.52 ± 2.40	19.37 ± 2.70
G3 Casein + DMH	11.84 ± 2.70	22.38 ± 2.70*
G4 Sericin + DMH	8.41 ± 3.00#	21.89 ± 2.70*#
G5 Post-sericin + DMH	12.37 ± 2.80	21.21 ± 1.00*

Note: Values are mean ± SD of 6 rats.

*$p \leq 0.05$, compared with casein diet (group 1)

#$p \leq 0.05$, compared with sericin diet (group 2)

Table 8 Effect of sericin on organ weight and serum enzymes in rat colons

General observations		G1 Casein	G2 Sericin	G3 Casein + DMH	G4 Sericin + DMH	G5 Post sericin + DMH
Rat organ weight (g)	Liver	15.47 ± 1.66	15.23 ± 1.37	14.77 ± 1.49	13.64 ± 0.48	13.82 ± 1.66
	Kidney	3.40 ± 0.37	3.25 ± 0.28	3.53 ± 0.25	3.02 ± 0.34	3.26 ± 0.35
	Heart	1.65 ± 0.23	1.64 ± 0.22	1.61 ± 0.09	1.55 ± 0.15	1.51 ± 0.15
	Small intestine	10.06 ± 1.08	10.33 ± 0.86	10.49 ± 1.20	10.06 ± 1.04	10.03 ± 1.22
	Spleen	1.06 ± 0.19	0.98 ± 0.11	1.13 ± 0.17	1.02 ± 0.04	1.00 ± 0.14
	Colon	1.70 ± 0.16	1.72 ± 0.30	1.96 ± 0.32	1.77± 0.19	1.58 ± 0.33
Serum enzymes	ALP	46.33 ± 4.80	47.50 ± 14.20	58.33 ± 10.46	48.83 ± 6.31	50.00 ± 10.45
	AST	224.83 ± 114.25	254.83 ± 132.29	391.17 ± 187.47	361.83 ± 265.31	233.50 ± 81.47
	ALT	59.33 ± 24.32	60.50 ± 20.60	185.17 ± 226.07	62.33 ± 10.91	58.00 ± 14.89
	LDH	2428.67 ± 680.99	2140 .17 ± 662.83	3210.17 ± 1011.38	2977.83 ± 800.58	2743.67 ± 578.74
	BUN	26.67 ± 2.80	25.00 ± 2.19	29.50 ± 3.89	25.00 ± 3.29	25.00 ± 2.97
	Creatinine	0.52 ± 0.12	0.48 ± 0.12	0.57 ± 0.12	0.50 ± 0.18	0.50 ± 0.18
	Uric acid	1.67 ± 0.43	1.83 ± 0.56	3.35 ± 2.70	1.83 ± 0.79	1.92 ± 0.49

2. Effect of sericin on incidence and distribution of aberrant crypt foci (ACF) and tumor in rat colons

The appearance of ACFs is considered to be an early sign of colon tumorigenesis that are widely used to identify as the earliest marker of carcinogen-induced colon cancer in rodents and to screen for potential chemopreventive agents [67]. ACF are morphologically abnormal crypts in the colonic mucosa identified by microscopic observation of colon stained with methylene blue. ACF were distinguished from normal crypts by their darker staining and large size, elliptical shape, thicker epithelial lining, and larger pericryptal zone [149]. In the present study, we determined the incidence and number of ACF in rat colon to evaluate the effect of sericin on colon tumorgenesis. In addition to ACF, tumor can be observed in rat colon.

The incidence of tumor formation and ACF incidence that occur in the colon of rats are summarized in Table 9. At the end of experiment, two from six rats in group 3 developed tumor (33%), but no tumor was observed in the sericin-fed rats (group 4 and 5). The results that showed ACF were formed in all DMH-treated rats (groups 3, 4 and 5), but there was no ACF formation in the colon of control rats (group 1 and 2). The number of ACF in sericin (group 4) and post-sericin (group 5) treatments were approximately 80 ACF/cm^2/rat which were lower than that of casein-fed rats (group 1). Although these reduction were not statistically significant. This result suggests that sericin tended to suppress the formation of ACF induced by DMH.

It is known that the number of ACF may reflect the initiation step of colon carcinogenesis. Consumption of the sericin containing diet may be potential to suppress or delay colonic tumorigenesis irrespective to whether intake was before or during the exposure of carcinogen.

Table 9 Effect of sericin on incidences and distribution of aberrant crypt foci (ACF) and tumor in rat colons

Treatment groups	No. of rats	ACF and tumor formation		
		Tumor Incidence (%)	ACF Incidence (%)	Number of ACF/cm^2/rat
G1 Casein	6	0	0	0
G2 Sericin	6	0	0	0
G3 Casein + DMH	6	2 (33)	6 (100)	116.01 ± 48.52
G4 Sericin + DMH	6	0	6 (100)	79.96 ± 29.04
G5 Post-sericin + DMH	6	0	6 (100)	82.35 ± 39.83

Note: Values are mean ± SD of 6 rats.

3. Density of aberrant crypt foci (ACF) in different parts of rat colons

The density of ACF (number/cm^2) in each part of rat colons; proximal, middle and distal were analysed as shown in Table 10. This study showed that ACFs were mostly distributed in the distal part of colon. This observation was correlated with the mechanism of DMH for induction of colon tumorigenesis. DMH is metabolized in the liver by conjugation with glucuronic acid and then enters the gut both with bile and directly via the circulation. In the gut, microbial beta-glucuronidase releases the active metabolite in turn which then can damage cells in surrounding area. Therefore, DMH specifically induces colon cancer especially in the distal colon where gut microbes are mostly localized [150, 151].

The results also showed that the density of ACF in casein-fed rats (group 3) was higher in all regions of colon than that of both sericin-fed rats (group 4 and 5). It should be noted that the casein-fed rats showed the ACF in the proximal part of colon, whereas there was no ACF in sericin-fed rats. Although these differences did not reach statistical significance, sericin consumption has potential to reduce the ACF formation in the colon.

Table 10 Density of ACF in differences parts of colon of DMH-treated rat

Treatment	Density of ACF (number/cm^2)			
	Total	**Proximal**	**Middle**	**Distal**
G3 Casein+DMH	116.01 ± 48.52	3.90 ± 5.30	37.99 ± 27.50	73.38 ± 27.02
G4 Sericin+DMH	79.96 ± 29.04	0	21.00 ± 18.47	57.79 ± 26.38
G5 Post-sericin+DMH	82.35 ± 39.83	0	28.35 ± 29.78	53.46 ± 22.78

Note: Values are mean ± SD of 6 rats.

4. Effect of sericin on crypt multiplicity of ACF

Generally, ACF was found to consist of 1, 2, 3, 4 or more crypts. It is believed that the number of crypts increases with time due to crypt multiplication or branching [152]. The crypts reproduce themselves by a fission mechanism [153]. However, what is still unresolved is the most relevant ACF parameter that will predict tumor incidence. Increased multiplicity has been suggested as a key parameter to use in order to predict tumor incidence or malignant transformation [154]. Therefore, a number of aberrant crypts in each focus (crypts/ACF) were analyzed in this study as shown in Table 11.

Since most ACFs were distributed in the distal colon, multiplicity of ACF in this part of colon was analyzed. ACF were divided into five categories depending on the number of crypts in each focus (1, 2, 3, 4 and $\geq$ 5 crypts/ACF). The results showed that the numbers of small ACF (1 and 2 crypts/ACF) were not different among three groups (Table 11). However, sericin-fed rats (group 4 and 5) showed the tendency to decrease their large ACF ($\geq$ 3 crypts/ACF) than casein-fed rats (group 3). Especially the largest ACF ($\geq$ 5 crypts/ACF) in group 5 significantly reduced comparing to casein-fed rats. It has been reported that large ACF containing at least 4 crypts per focus are more likely to progress into tumors [155]. The progressive increase in the number of crypts per ACF may correspond to the promotion step of colon carcinogenesis [156]. Therefore, sericin treatment seems to be a potential agent to inhibit the progression of colon cancer in the promotion stage since the numbers of large ACFs were found to be lower than those of casein-fed rats.

Table 11 Crypt multiplicity of ACF in DMH-treated rat colons

Type of ACF in distal colon	ACF (number/cm^2)		
	Casein + DMH	Sericin + DMH	Post-sericin + DMH
1 crypt/ ACF	6.49 ± 6.27	6.06 ± 7.69	5.84 ± 6.96
2 crypts/ ACF	12.01 ± 8.70	12.34 ± 10.48	11.26 ± 7.19
3 crypts/ ACF	9.74 ± 6.49	7.58 ± 6.49	9.52 ± 7.47
4 crypts/ ACF	7.47 ± 7.14	3.68 ± 1.73	5.41 ± 4.89
≥ 5 crypts/ ACF	37.66 ± 10.12	28.79 ± 15.79	21.43 ± 9.03*

Note: Values are mean ± SD of 6 rats.

*$p \leq 0.05$ compared to casein + DMH group

5. Area analysis of large size ACF

Because it was not readily to distinguish individual crypts in the largest ACFs (≥ 5 crypts/ACF), we instead measured their surface area. Table 12 shows the average size or surface area of large ACF in middle and distal colon. In the middle part of colon, no statistically significant difference was observed among DMH-treated rats (group 3, 4 and 5). In the distal part of colon, it is interesting to note that the large size ACF of casein-fed rats (group 3) was 0.14±0.06 mm^2, while smaller ACF (0.11±0.05 mm^2) was observed in rats fed sericin diet (group 4). Taking all data together, sericin consumption, prior to carcinogen exposure, appears to reduce ACF density as well as crypt multiplicity suggesting that the sericin diet suppresses both ACF formation and progression.

Table 12 Area analysis of large size ACF (≥ 5 crypts per focus) in DMH-treated rat colons

Treatment groups	Middle		Distal	
	Number of ACF [b]	Area of each ACF [a] (mm^2)	Number of ACF [b]	Area of each ACF [a] (mm^2)
G3 Casein + DMH	54.00 ± 14.20	0.09 ± 0.07	116.00 ± 7.79	0.14 ± 0.06
G4 Sericin + DMH	37.00 ± 7.73	0.09 ± 0.08	133.00 ± 12.16	0.11 ± 0.05*
G5 Post-sericin + DMH	50.00 ± 9.05	0.10 ± 0.06	99.00 ± 6.95	0.14 ± 0.06

Note: [a] Values are mean ± SD of 6 rats.

[b] Values are total ACF from 6 rat ± SD

* $p \leq 0.05$ compared to casein + DMH group

6. Effect of sericin on lipid peroxidation in rat colons

The production of malondialdehyde (MDA), the end product of lipid peroxidation was measured as shown in Table 13. The results showed that there was no significant difference in the level of lipid peroxidation in all parts of colon among all groups. The proximal region of all rats showed similar level of lipid peroxidation. In the middle region, the levels of lipid peroxidation of sericin-fed rats were slightly higher than casein-fed rats. The results showed that sericin consumption either prior or during DMH treatment seemed to reduce lipid peroxiadtion product in distal colons compared to that of casein diets, although this difference did not statistically significance. The result from this study, particularly in distal colons, suggests the anti-oxidative effect within the intestinal tract of sericin diet.

DMH is metabolized to a methyl radical which with metal ions generates hydroxyl radical or hydrogen peroxide which caused lipid peroxidation [157]. Several studies also reported that MDA levels were increased in cancerous tissues when compared to healthy controls [158, 159]. In animal model, lipid peroxidation was found to increase significantly in the plasma and red blood cells of DMH treatment when compared to control rats [160]. A sericin diet has previously been shown to reduce oxidative stress in the colonic mucosa that might, at least in part, lead to reduction of cell proliferation [9]. In this study, sericin diet tended to lower the levels of MDA than casein diet particulaly in distal region. The present study suggests that sericin with its anti-oxidant property can suppress the ACF formation probably by reducing the level of lipid peroxidation in colon.

Table 13 Effect of sericin on lipid peroxidation in rat colons

Treatment groups	MDA concentration (μM)		
	Proximal	**Middle**	**Distal**
G1 Casein	1.06 ± 0.40	0.88 ± 0.22	1.26 ± 0.51
G2 Sericin	1.13 ± 0.59	0.99 ± 0.29	0.81 ± 0.21
G3 Casein + DMH	1.07 ± 0.38	0.64 ± 0.22	1.77 ± 1.26
G4 Sericin + DMH	1.06 ± 0.22	1.75 ± 1.05	1.08 ± 0.71
G5 Post-sericin + DMH	1.08 ± 0.52	1.16 ± 0.40	1.15 ± 0.64

Note: Values are mean ± SD of 6 rats.

7. Immunohistochemical analysis of Ki67 in rat colons

Because the mechanisms involved in protective effects of sericin against colon tumorigenesis are not clearly understood, the effect of sericin on cell proliferation was investigated in this study. Cell proliferation is believed to be related to the risk of colon cancer. Higher cell proliferation and expansion of the proliferative zone in colon mucosa were associated with higher risk of colon cancer [161]. In this study, cell proliferation was evaluated by analysing Ki67, a known cell proliferation marker.

Ki67 protein was used in view of its sensitivity as a marker of cell proliferation since it is expressed in all phases of the cell cycle except in resting phase [95]. Thus the proliferating colonic cells expressed Ki67 protein in the nucleus could be found in the colonic crypts. The number of Ki67-positive cells found in colonic crypt is shown in Table 14. In the present study, all parts of colon segments, normal casein-fed rats (group 1) had lesser number of Ki67-positive cells than that of normal sericin-fed rats (group 2). The results may indicate that sericin could partially accelerate cell proliferation in normal state of colonic crypt cells. In the present study, most parts of colon segments in DMH-treated rats (group 3, 4 and 5) showed higher number of Ki67-positive cells than those of normal rats (group 1 and 2).

Among DMH-treated rats, sericin-fed rats (group 4 and 5) showed more number of Ki67-positive cells in the colonic crypt than that of casein-fed rats (group 3) (Table 14). Significant increase in Ki67-positive cells in distal parts was found only in group 5. From this result within either normal or DMH-treated rats, sericin supplement seemed to slightly increase the numbers of Ki67-positive cells in colonic crypts. Although there is no evidence to support this, it is possible that sericin might accelerate colonic cell proliferation to speed up colonic cells to be shed off or to renew the mucosa layer.

In addition to number of Ki67-positive cells, the length of colonic crypt was measured to evaluate the proliferation phase. The results showed that the length of colonic crypt in normal control rats (group 1 and 2) did not differ in proximal part (Table 15). Compared with the casein-fed rats (group 1), sericin-fed rats (group 2) showed a slight increase in length of colonic crypts in middle and distal parts. However, no significant difference was found. In DMH-treated rats, the length of

colonic crypts of sericin-fed rats (group 4 and 5) were higher than that of the casein-fed rats (group 3). The length of colonic crypts in distal parts of group 5 rats (consumption of sericin during DMH exposure) was significantly increased. Although the explanation of this phenomenon is not clear, this data correspond to Ki67 analysis that sericin might accelerate colonic crypt cell proliferation and then led to the increased length of colonic crypt. It should be noted that colonic crypts that were selected for these particular experiments are normal looking crypts where colonic cells normally align from the base to luminal surface of crypts. It is impossible to quantitatively analyze Ki67 in ACF crypts because very little chance of cross-sectioning into these specific areas and crypts in these areas appear in irregular in shape and size.

In the present study, chemopreventive effect of sericin on colon carcinogenesis can not simply explain by the mechanism through cell proliferation. Therefore, other mechanisms such as apoptotic process may be involved in the effect of sericin. With respect to Bcl-2, the increased of Bcl-2 expression occasionally observed in transitional mucosal might indicate that decreased apoptosis could be one of the mechanisms responsible for the increased crypt length [162].

Table 14 Total number of Ki67 positive cells in colonic crypt

Treatment groups	Total number of Ki67 positive cells		
	Proximal	Middle	Distal
G1 Casein	26.03 ± 12.48	28.90 ± 1.87	15.78 ± 3.00
G2 Sericin	27.67 ± 7.20	35.13 ± 3.36	18.25 ± 6.97
G3 Casein + DMH	27.60 ± 6.18	33.03 ± 5.33	17.08 ± 4.42
G4 Sericin + DMH	30.27 ± 5.72	37.20 ± 6.52	28.55 ± 10.33
G5 Post-sericin + DMH	27.61 ± 7.78	31.97 ± 5.42	29.82 ± 9.55*

Note: Values are mean ± SD of 6 rats.

*$p \leq 0.05$ compared to casein + DMH group

Table 15 Length of colonic crypts

Treatment groups	Length of crypt (μm)		
	Proximal	Middle	Distal
G1 Casein	210.17 ± 16.50	268.31 ± 32.25	220.62 ± 34.90
G2 Sericin	222.18 ± 14.08	311.41 ± 25.80	228.64 ± 34.51
G3 Casein + DMH	240.02 ± 14.45	287.05 ± 46.27	224.42 ± 19.55
G4 Sericin + DMH	271.25 ± 53.82	337.45 ± 61.19	237.30 ± 46.67
G5 Post-sericin + DMH	261.75 ± 42.32	302.08 ± 25.87	270.08 ± 13.83*

Note: Values are mean ± SD of 6 rats.

*$p \leq 0.05$ compared to casein + DMH group

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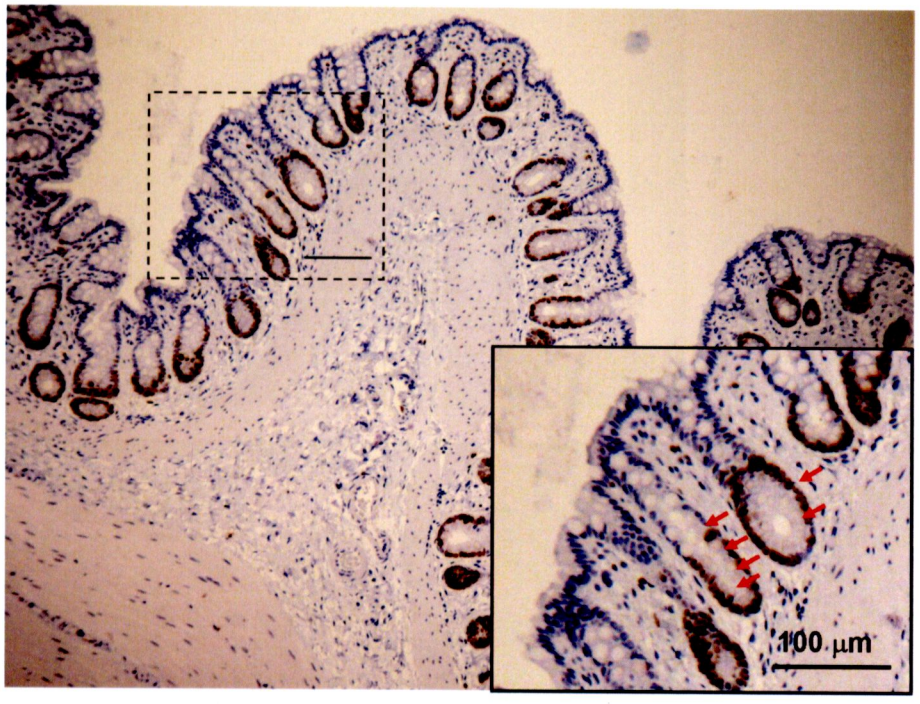

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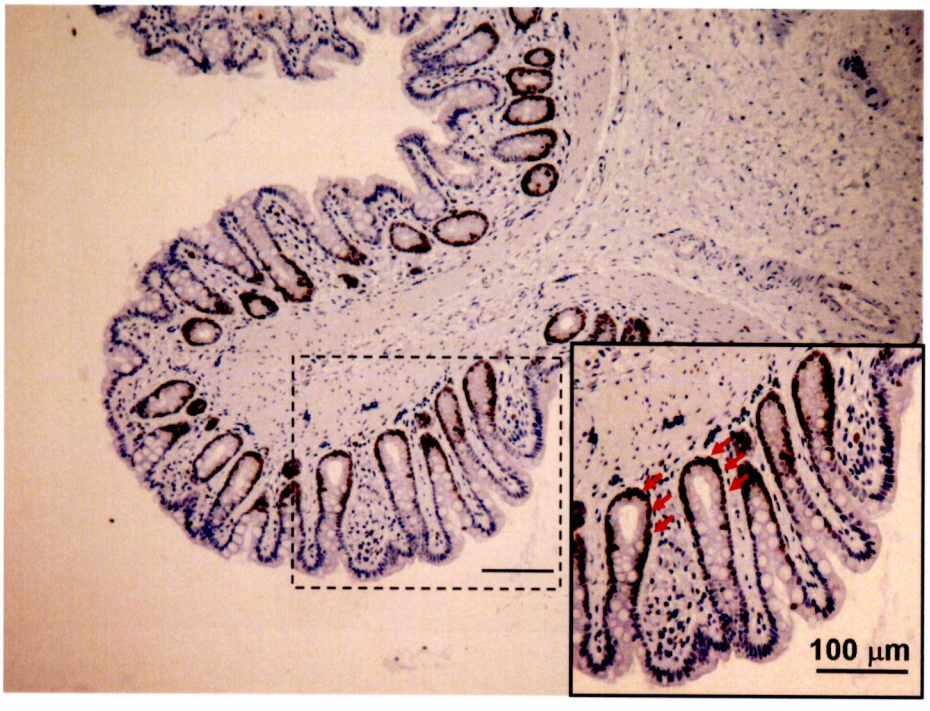
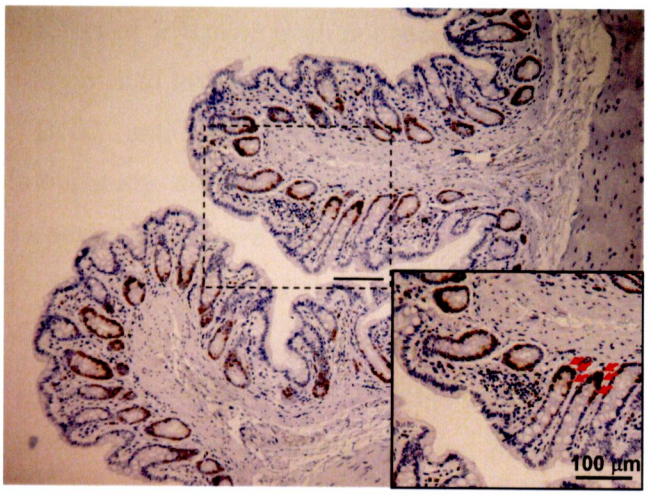


Figure 16 Immunohistochemical analysis of Ki-67 expression in distal colon of rat. (A) control casein-fed rats (group 1) and (B) control sericin-fed rats; Red arrow: Ki67-positive cells.

A



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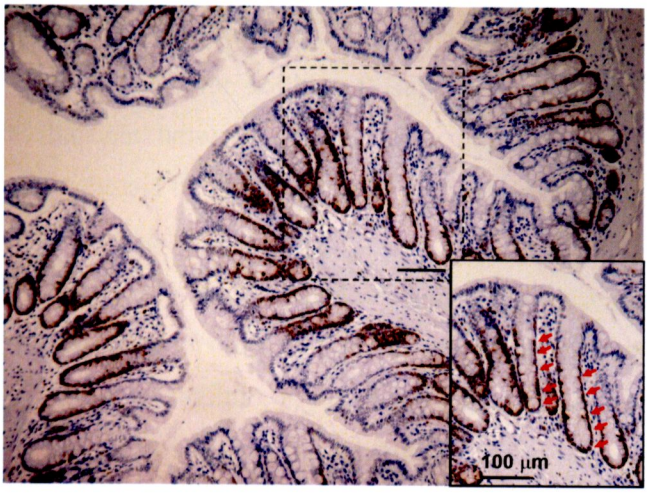

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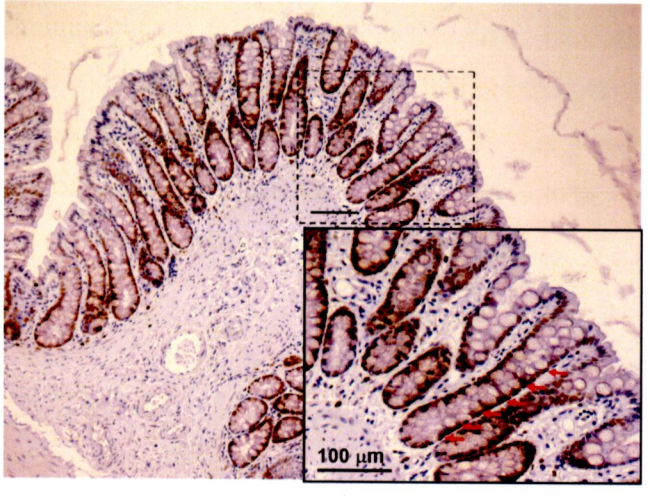


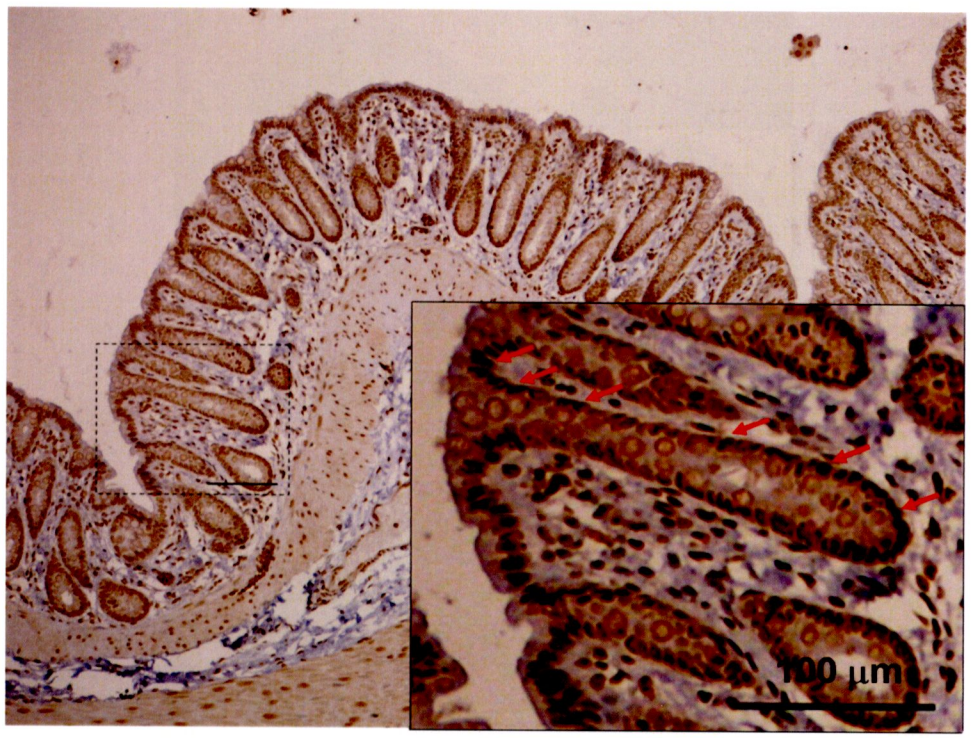
Figure 17 Immunohistochemical analysis of Ki-67 expression in distal colon of DMH-treated rats. (A) casein-fed rats (group 3), (B) sericin-fed rats (group 4) and post-sericin fed rats (group 5); Red arrow: Ki-67-positive cells.

8. Effect of sericin on immunohistochemical analysis of Bcl-2

In order to investigate the mechanisms by which sericin supplement evoke apoptosis, Bcl-2 anti-apoptotic protein was analyzed by immunohistochemical analysis. In our study, the expression of Bcl-2 found in most cells throughout colonic crypts and unexpectedly there was no significant difference between normal control rats (Figure 18) and DMH-treated rats (Figure 19). Bcl-2 normally localizes to the inner mitochondrial membrane and it produces dramatic extension of cell survival when it is over-expressed [87]. Thus, Bcl-2 is usually highly expressed at the base of colonic crypts cells which presumed location of stem cells in normal condition, suggesting that it protects stem cells from apoptosis [87]. If its expression was observed other than the base of colonic crypts, it would therefore be considered abnormal state [163]. The ability of Bcl-2 to inhibit apoptosis depends on the intracellular balance between anti-apoptotic and pro-apoptotic protein [164]. The inhibition of apoptosis by Bcl-2 is considered to occur through its interactions with a pro-apoptotic homologue, Bax [89].

In the present study, Bcl-2 staining cells were appeared throughout the colonic crypt in normal rats (group 1 and 2) as same as in the DMH-treated rats (group 3, 4 and 5). Bcl-2 is an inhibitor of apoptosis and, it should be mostly expressed in the basal area of the colonic crypts. In this study, anti-Bcl-2 antibody used here failed to distinguish the difference among cells along colonic crypts. We have tried to adjust staining technique but the result came out the same. This antibody however worked well in positive control section of rat spleen. It is possible that this anti-Bcl-2 antibody might cross-react with other Bcl-2 family, such as Bax protein. Therefore, the result from this set of experiment (Bcl-2 staining) can not be used for explaining the mechanism of chemopreventive effect of sericin diet.

A



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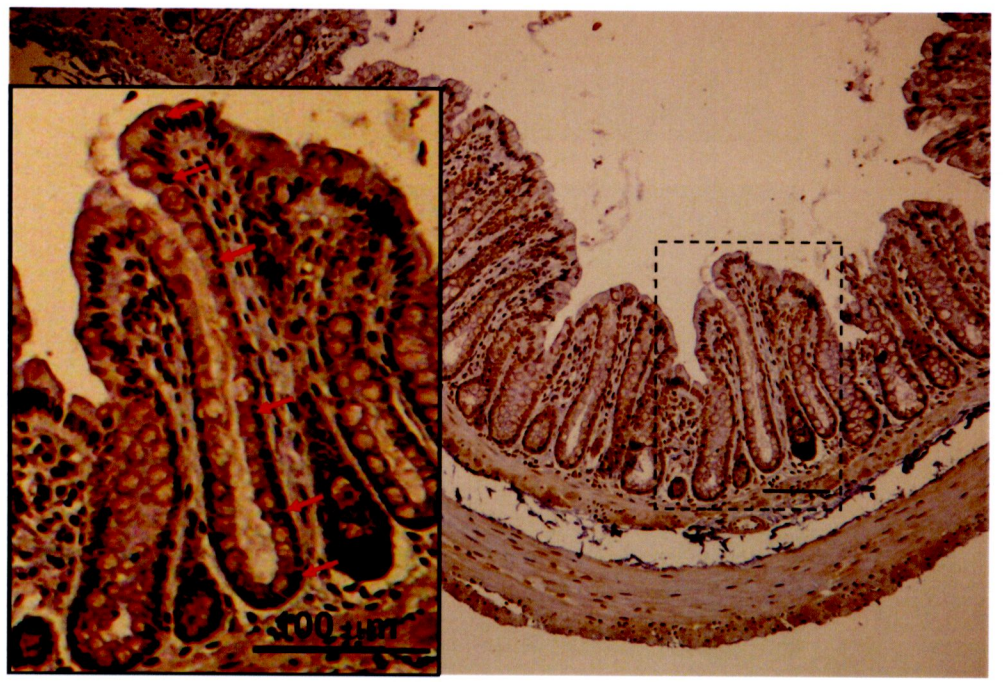
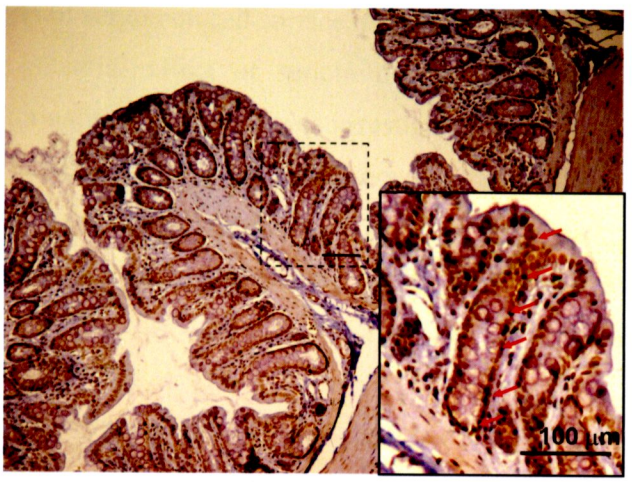


Figure 18 Immunohistochemical analysis of Bcl-2 expression in distal colon of control rat. (A) control casein-fed rats (group 1) and (B) control sericin-fed rats; Red arrow: Bcl-2-positive cells.

A



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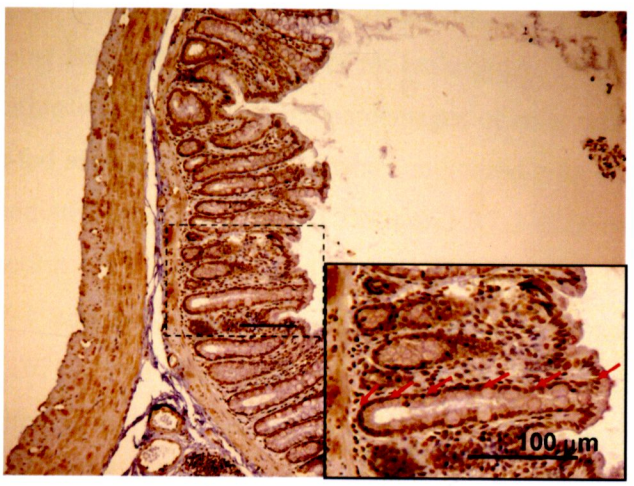

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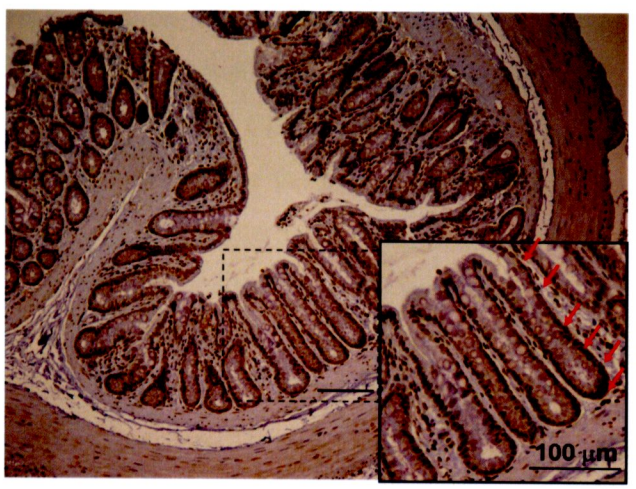

Figure 19 Immunohistochemical analysis of Bcl-2 expression in distal colon of DMH-treated rats. (A) casein-fed rats (group 3), (B) sericin-fed rats (group 4) and post-sericin fed rats (group 5); Red arrow: Bcl-2-positive cells.

9. Effects of sericin on cell surface markers in rat blood

The potential effect of sericin on systemic immunity that might be implicated in eliminating tumor cells was investigated. The immune cell profile of the rats was analyzed based on cell surface markers of peripheral blood leukocytes. At the end of experiment, the blood samples were obtained from rats by cardiac puncture. The peripheral blood leukocytes were stained with antibodies specific for CD2, CD3, CD4, CD8a, CD11a, CD25, CD45, CD54, CD80 and CD86 and then analyzed using flow cytometer (Figure 20).

It has been reported that diet plays an important role in cancer prevention by modifying the activities of protective systems, including immune system [165]. Our present observations found that there was no difference among the effect of casein and sericin diet on most cell surface markers of rat leukocytes. The significant reduction in immune activation was observed in the percentages of cells stained with CD8a and CD80 in the rats fed sericin diet (group 2) when compared with casein diet (group 1). Costimulatory mediated molecules B7-1 (CD80) and B7-2 (CD86) play an important role in the induction of T cell-mediated anti-tumor immunity [166]. Ohtani et al. reported that B7-1 and B7-2 was increased in peritumoral macrophages of colon cancer patients [167]. CD8a is a surface marker of cytotoxic T cells and NK cells, which are responsible for immune response in elimination of tumor cells. In the present study, there was a reduction of the percentages of $CD8a^+$ cells, whereas there was no significant alteration in $CD4^+$ cells. These results are contrary to an observation in a human study showing that $CD4^+$ and $CD8^+$ T cells confer anti-tumor activities. An increase in peripheral $CD8^+$ T cells and a decrease in $CD4^+$ T cells are associated with tumor progression in the human study [168]. However, at the later stage of tumor formation, the malignant tumor may have developed mechanisms that inhibit immune function [169] or the immunosuppression may be nonspecific due to other physiologic factor or malnutrition [170]. It should be noted that rats in group 1 and 2 are normal rats therefore it is difficult to draw a conclusion regarding to the changes in these surface markers. However, the mechanism underlies the reduced $CD8a^+$ and $CD80^+$ cells in this present study is not known, but this might result from different amino acid compositions in these diets that may have an impact on these

reductions. Further studies are needed to clarify the mechanisms involved in immune regulation of sericin protein.

In DMH-induced colon cancer, there was a little change in the pattern of rat peripheral blood leukocytes when compared with normal rats. However, the results showed a significant decrease in $CD8a^+$ cells in DMH-treated rats of groups 3, 4, and 5, a significant increase in $CD3^+$ and $CD25^+$ cells in rats fed with sericin diet (group 4), and a slight increase in $CD11a^+$ cells in DMH-treated rats of groups 3 and 4. It has been reported that $CD4^+$ T cells obtained from the intestine of patients with intestinal inflammation have a higher adhesion molecule CD11a expression than healthy subjects [171]. In this present study, CD11a were found in normal rats and this adhesion molecule slightly increased in DMH-treated rats. This finding might be due to an up-regulation of immune cell interactions via CD11a [172]. Among DMH-treated groups, sericin diet (group 4) showed a significant increase in $CD2^+$, $CD3^+$ and $CD25^+$ cells compared with casein diet (group 3). The increase in $CD2^+$ cells in the present study is in accordance with a previous study on the percentages of activated lymphocytes in the tumor infiltrating lymphocytes (TIL) isolated from colon cancer patients [173]. The results suggest that in the presence of CD2-positvie T cells, NK cells and dendritic cells may be activated by foreign surface determinants on tumor cells, particularly in sericin supplement.

T cells ($CD3^+$) consisting of $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells are believe to play an important role in immune responses to tumors [104]. However, the T cells response to tumor is usually impaired, with defects at various levels of the adaptive response: a predominance of T helper ($CD4^+$) response is common and leads to a decrease in activated cytotoxic T cells ($CD8^+$) [174]. Pierre et al. have reported that T cell status changes during tumor formation in mice fed with food supplemented with short chain fructo-ologosaccharides [175]. This present study is consistent with their study, as increased number of $CD3^+$ T cells were found, which might result from increased number of $CD4^+$ T helper cells. Moreover, the increased $CD3^+$ T cells were activated (having CD25 on the cell surface), suggesting that sericin can activate immune response in colon carcinogenesis.

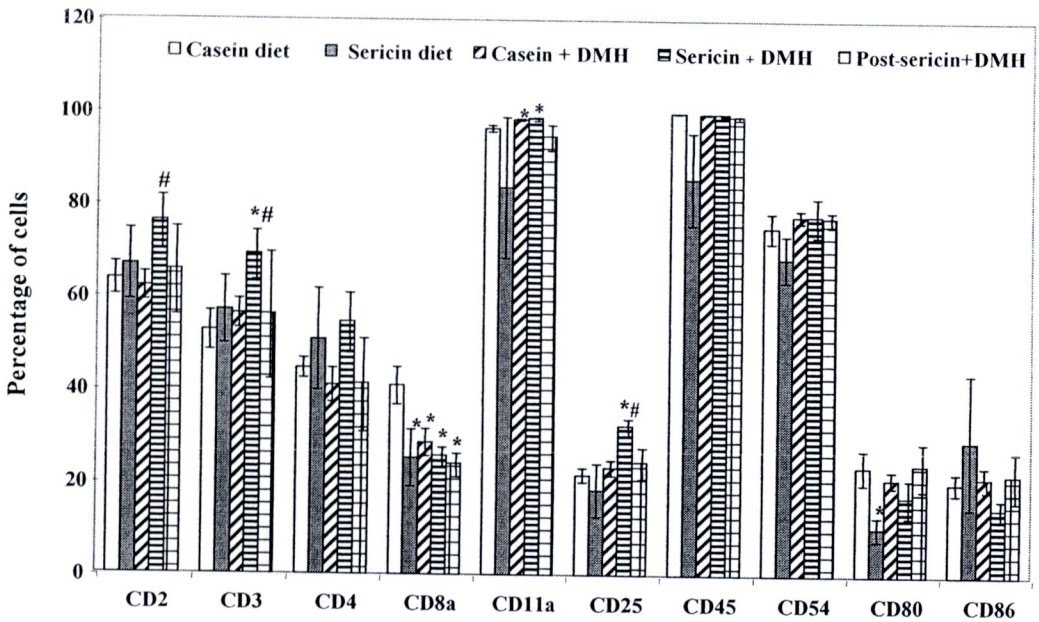

Figure 20 Flow cytometry of rat surface markers.

Note: Rat blood leukocytes were stained with antibodies specific for CD2, CD3, CD4, CD8a, CD11a, CD25, CD45, CD54, CD80 and CD86 and analysis using flow cytometer. Values are mean ± SD of 6 rats. * $p \leq 0.05$ compared to casein diet (group 1) $^{\#}$ $p \leq 0.05$ compared to casein + DMH (group 3).