STUDIES ON THE EFFECTS OF GINSENOSIDES Rg_1 AND Rb_1 OF *PANAX GINSENG* ON MITOSIS

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Introduction

The dry root of ginseng (*Panax ginseng* C.A. Meyer) has long been used as a tonic. It is particularly valued by older people for its palliative effect on degenerative conditions.

Ginsenosides (ginseng saponins) have been reported to be the most important biological components of ginseng (Oura *et al.*, 1975) and among about 15 different kinds of ginsenosides, Rg_1 and Rb_1 are considered as the two main ones. These two ginsenosides show some opposite pharmacological effects, i. e. Rg_1 has stimulating effect on central nervous system and Rb_1 has suppressing effect on it (Shibata and Saito, 1977). However, reports of thier biochemical effects in animals are not consistant. Rg_1 was shown to be able to accelerate DNA, protein and lipid synthesis in rat bone marrows *in vivo* (Yamamoto *et al.*, 1974) and to increase leucine incorporation into mouse sera (Oura *et al.*, 1975) but Rb_1 could not. On the other hand, Shibata *et al.* (1976) and Iijima *et al.* (1976) reported that Rb_1 could promote serum protein and RNA synthesis respectively in rat *in vivo* but Rg_1 could not. In addition, it had been demonstrated that the administration of Rb_1 to rats could promote the cholesterol synthesis in liver, but *in vitro* direct administration to rat liver slices showed no such effect (Gommori *et al.*, 1976).

The normal human lymphocytes in the peripheral blood do not divide. However, when such lymphocytes are activated by a mitogenic lectin, such as phytohemagglutinin(PHA)(Nowell, 1960), they can undergo a series of changes and divide within 72 hours. In this paper, we report the effects of Rg_1 on mitosis, cell density and DNA synthesis in cultured human lymphocytes in the presence or absence of a mitogenic lectin.

Meanwhile, although ginseng is a plant product, its action on plant cells has not been studied. The structure and physiology of plant cells are quite different from that of animals. The study of the effects of ginsenosides on plant cells may provide useful information about their action. Thus, the effects of Rg_1 and Rb_1 on mitosis and durations of mitotic cell cycle and S period in root tip cells, as well as DNA synthesis in seedlings of *Allium cepa* were also investigated.

Effects of Rg_1 on mitosis in human blood lymphocytes

Materials and Methods

In this study, blood from the same donor was used throughout. Peripheral blood lymphocy-

tes were grown in TC Chromosome Microtest Medium (Difco Laboratories, U.S.A.) which contains PHA. We followed the most procedures provided by the manufacturer for blood culture and slide preparation. The study of the effects of Rg_1 in the absence of a mitogenic agent was carried out using Eagle's MEM (Gibco, U.S.A.). In this case, the cell suspension was prepared according to the method of Waithe and Hirschhorn (1978). Rg_1 was first dissolved in sterile 0.1 M phosphate buffer, pH 7.4, and added to the medium. Equal amount of the buffer was added to the control.

1. Effect of Rg_1 on mitosis——Graded concentrations of Rg_1 were added to the culture medium on the 3rd day. The cells were fixed on the 4th day and stained with acetocarmine. Mitotic index
$$(MI = \frac{\text{No. of dividing cells}}{\text{Total No. of cells counted}} \times 1000)$$
and percentages of different mitotic stages were determined for each treatment from about 2500 cells by scoring 500 cells for each of 5 slides.
2. Effect of Rg_1 on cell density——Rg_1 was added to the medium just before starting the cell culture. Number of cells was counted by the haemacytometer method (Kuchler, 1977) on different days of culture until 21st day.
3. Effect of Rg_1 on cell density in the presence and absence of a mitogenic lectin, concanavalin A (Con A)——The medium, MEM was supplemented with 0.125 ml of heat-inactivated fetal calf serum, 2 μg of Fungizone, 100 units of penicillin and 0.1 mg of streptomycin per ml of medium (Kuchler, 1977). Four different treatments were performed.
4. Effect of Rg_1 on DNA synthesis——Rg_1 (0.0005 mg/ml) was added on the 3rd day of culture. ^{3}H-thymidine (^{3}H-TdR, specific activity 2.0 Ci/mM, Radiochemical Center, England) was added to two separate cultures at 2.5μ Ci/ml for the last 15 and 7 hours of incubation period respectively. This experiment was repeated once. Cells were collected on 0.45 μm Millipore filters and the incorporation of ^{3}H-TdR into the cells was measured by a Backman liquid scintillation counter (Diamantstein and Ulmer, 1975).

In the study of the effect of Rg_1 on DNA synthesis in the presence of N-acetylgalactosamine (NAGAL, Borberg *et al.*, 1968), both chemicals were added to the medium before the inoculation of blood cells. ^{3}H-TdR was added on the 3rd day of culture (last 15 hours of incubation period).

Results

Effect of Rg_1 on mitosis

Three experiments were carried out. In the first one, 4 concentrations of Rg_1 were used. The cells were fixed on the 4th day of culture at 11:00, 12:30, 14:00 and 15:30 (24 to 28 hours after the addition of Rg_1). The results (Fig. 1) indicate that (1) mitotic indices of the Rg_1-treated lymphocytes are significantly higher than that of the control in all treatments, (2) the highest MI is seen at 0.0005 mg/ml concentration and (3) from the distribution of cells in different mitotic stages, it is apparent that Rg_1 does not arrest dividing cells at any particular stage. The results of the second experiment (6 concentrations of Rg_1 were used) are in agreement with that of the first one (Fig. 2).

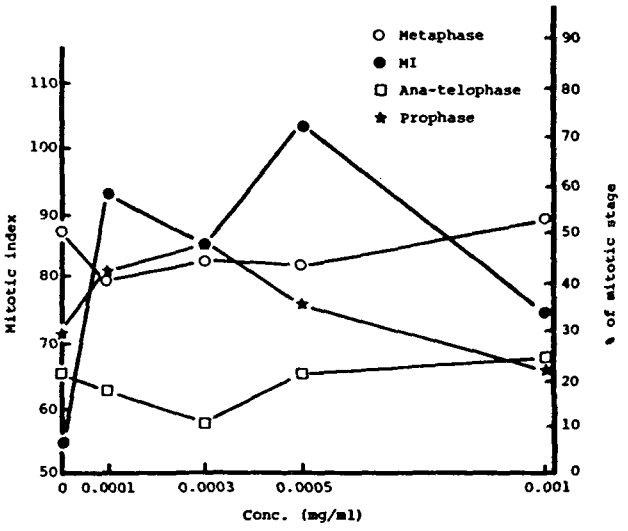

Fig. 1. The mean dose effect (average of 4 time periods) of Rg_1 on mitosis in human lymphocytes *in vitro* (experiment 1).

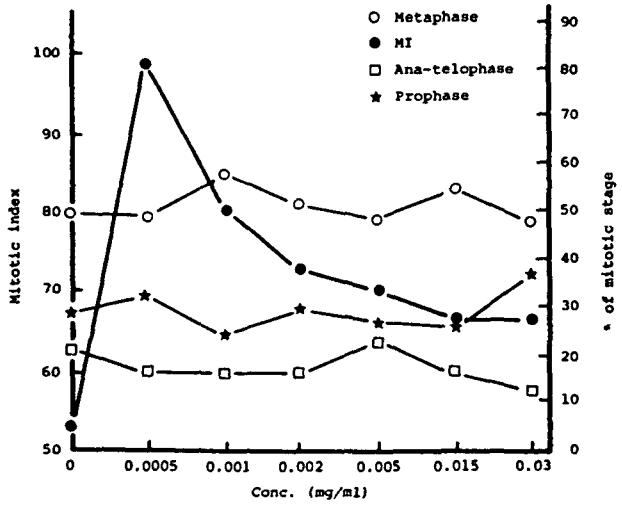

Fig. 2. The mean dose effect (average of 4 time periods) of Rg_1 on mitosis in human lymphocytes *in vitro* (experiment 2)

The third experiment was carried out to determine the effect of Rg_1 (0.0005 mg/ml) on mitosis at the earlier and later time periods than those in previous experiments, namely 12, 13 1/2 and 26 hours after the addition of Rg_1. Highly significant differences ($p < 0.001$) in MI were observed between the treated and control lymphocytes in all 3 cases (Table 1). The stimulatory effect of Rg_1 on mitosis is evident even within 12 hours of treatment.

Effect of Rg_1 on cell density

The cell densities of both the treated (0.0005 mg Rg_1/ml) and the control cultures were determined from the 5th day of culture onward until the death of cells (on the 21st day of culture). The cell numbers are significantly greater in the treated than in the control cultures on all different days (Fig. 3).

Table 1. Mitotic index in cultured human lymphocytes treated with Rg_1 (0.0005 mg/ml) at 3 different time periods on the 4th day of culture (Experiment 3).

Time period	Mitotic index (mean ± S.D.)		
Treatment	0:00	1:30	14:00
Control	56.40 ± 1.35	63.14 ± 2.10	67.85 ± 1.73
Rg_1	97.71 ± 0.85***	87.23 ± 1.12***	103.98 ± 1.42***

*** $P < 0.001$

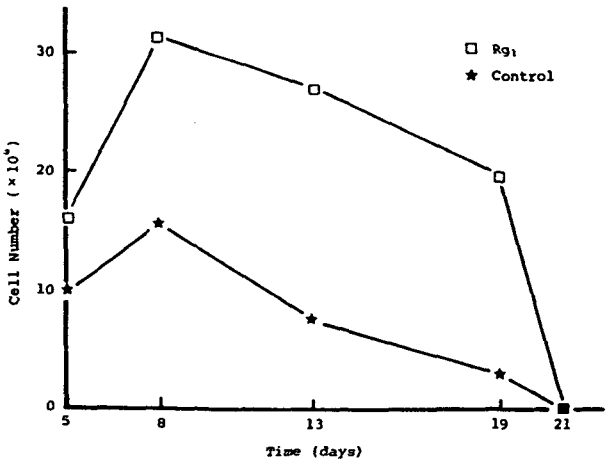

Fig. 3. Effect of Rg_1 (0.0005 mg/ml) on cell density of cultured human lymphocytes.

Effect of Rg_1 on cell density in the presence and absence of Con A

Cell densities were determined from the 1st to 26th day after culture in the following treatments: (a) the control (MEM only), (b) Con A-treated (0.05 mg/ml, Speckart *et al.*, 1978), (c) Con A-Rg_1-treated (0.05 mg of Con A/ml, 0.0005 mg of Rg_1/ml) and (d) Rg_1-treated (0.0001, 0.0003, 0.0005 and 0.001 mg/ml).

Cell densities of the Con A-treated cultures are significantly greater than that of the control from the 4th day to 10th day (Fig. 4). However, the differences in cell densities between the Con A-Rg_1-treated and the control are much more significant from the 4th day to the 26th day. On the other hand, the cell densities of the Rg_1-treated cultures (4 different concentrations) show no significant difference with that of the control.

Effect of Rg_1 on DNA synthesis

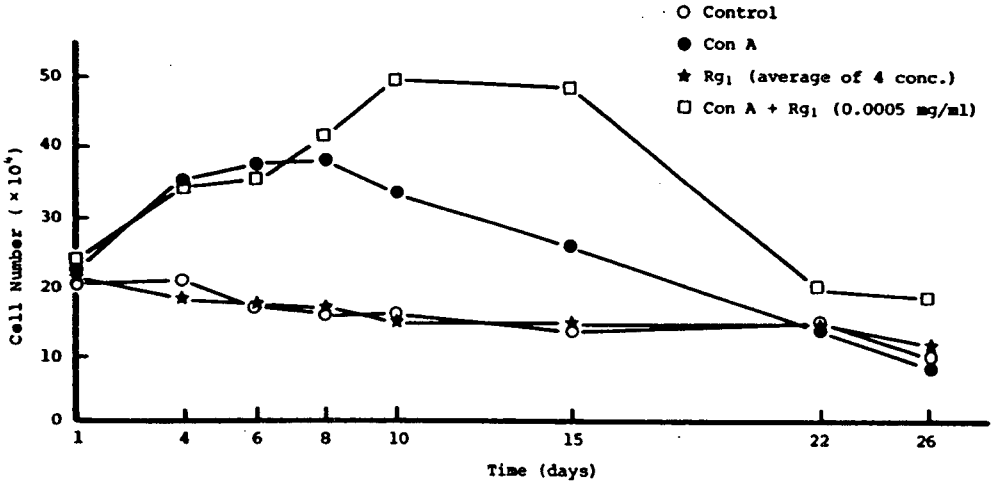

Fig. 4. Effect of Rg_1 on cell density of human lymphocytes in culture in the absence and presence of Con A (0.05 mg/ml).

Table 2. Effect of Rg_1 (0.0005 mg/ml) on DNA synthesis in cultured human lymphocytes (measured by the uptake of ^{3}H-TdR)

Experiment	Treatment	^{3}H-TdR uptake (cpm/culture)
1	23:00 on 3rd day	
	Control	647810
	Rg_1	662160
	7:30 on 4th day	310455
	control	310455
	Rg_1	223520
2	23:00 on 3rd day	
	Control	224890
	Rg_1	269450
	7:30 on 4th day	
	Control	263810
	Rg_1	163770

The uptake of ^{3}H-TdR was much higher in the Rg_1-treated lymphocytes than in the control (Table 2) when the tracer was added at 23:00 on the 3rd day of culture (12 hours after the addition of Rg_1 and 15 hours before harvesting the cells). The reverse is true when the ^{3}H-TdR was added at 7:30 on the 4th day of culture (20 hours after the addition of Rg_1 and 7 hours before harvesting the cells). From these results, it appears that the time of active DNA synthesis is different in Rg_1-treated and untreated lymphocytes.

The effect of Rg_1 on DNA synthesis in lymphocytes was further studied by the addition of NAGAL to the culture medium. When only NAGAL (10.0 mg/ml) was added, ^{3}H-TdR uptake by lymphocytes was significantly reduced (Table 3). However, the additions of Rg_1 (0.0005 mg/ml) together with NAGAL to the culture medium restored the ^{3}H-TdR uptake by lymphocytes.

Table 3. Effect of Rg_1 (0.0005 mg/ml) on DNA synthesis in cultured human lymphocytes in the presence of NAGAL (10 mg/ml)

Treatment	^{3}H-TdR uptake (cpm/culture)
Control	106400
NAGAL	68635
NAGAL + Rg_1	101939

Effects of Rg_1 and Rb_1 on mitosis in root tip cells of *Allium cepa*

Materials and Methods

Bulbs of *Allium cepa* were secured locally. When the roots (cultured in water) reached 1.5–2.0 cm long, individual bulbs were carefully screened for their uniformity and then randomized for treatment. Sterilized onion seeds (Taxas Early Yellow Grano 502, Dessert Seed Company, U.S.A.) were allowed to germinate on moist filter papers in petri dishes in an incubator at 25°C. Seedlings were selected for treatment when the roots were 1.0–1.5 cm long. Rg_1 and Rb_1 were dissolved separately in distilled water. All experiments were carried out in a laboratory at around 25°C.

1. Concentration and time course effect

In the study of the concentration effect, both bulb and seedling roots were treated with graded concentrations of Rg_1 and Rb_1 for 24 hours. In the study of the time course effect, seedling roots were treated with Rg_1 and Rb_1, both at 0.004 mg/ml, and fixed after 0, 3, 6, 10, 18, and 24 hours of treatment. Control roots were grown in distilled water. The roots were fixed in acetic alcohol (1:3) and stained with Feulgen reagent. Five roots were used for each concentration or duration and about 1000 cells were scored in each root.

2. Effect on durations of mitotic cycle and S period

The determination of mitotic cycle and S period duration was based on methods developed by Quastler and Sherman (1959), Van't Hof (1965 a,b) and Kuroki and Tanaka (1973). Roots of 3 bulbs were pulse labeled with ^{3}H-TdR at 4 μCi/ml for 30 min. After washing, they were allowed to grow in distilled water, Rg_1 and Rb_1 solution (both at 0.004 mg/ml) separately. Samples of 1–2 roots of each bulb were fixed at intervals of 2 hours up to 24 hours after pulse labeling. Slides (Feulgen preparations) were coated with Kodak NTB_2 emulsion and exposed for 10 days. Proportions of labeled cells at early prophase and metaphase were determined from over 1000 labeled and non-labeled cells at different mitotic stages in each root.

3. Time course effect on the rate of DNA synthesis in seedlings

One hundred seedlings were selected for each sampling time. After the removal of the tiny seed coat together with the haustorial tip of cotyledon and endosperm, they were weighed and treated with Rg_1 or Rb_1 at 0.004 mg/ml for 0, 3, 6, 10, 18 and 24 hours. Seedlings were labeled with ^{3}H-TdR at 0.5 μCi/ml for the last 2 hours of treatment (Furmanowa and Oledzka, 1978). They were then fixed with buffered neutral formalin at 4°C. We followed the most procedures of Bloch *et al.* (1967) for DNA extraction and counting of the radioactivity. Duplicate samples were used for each treatment.

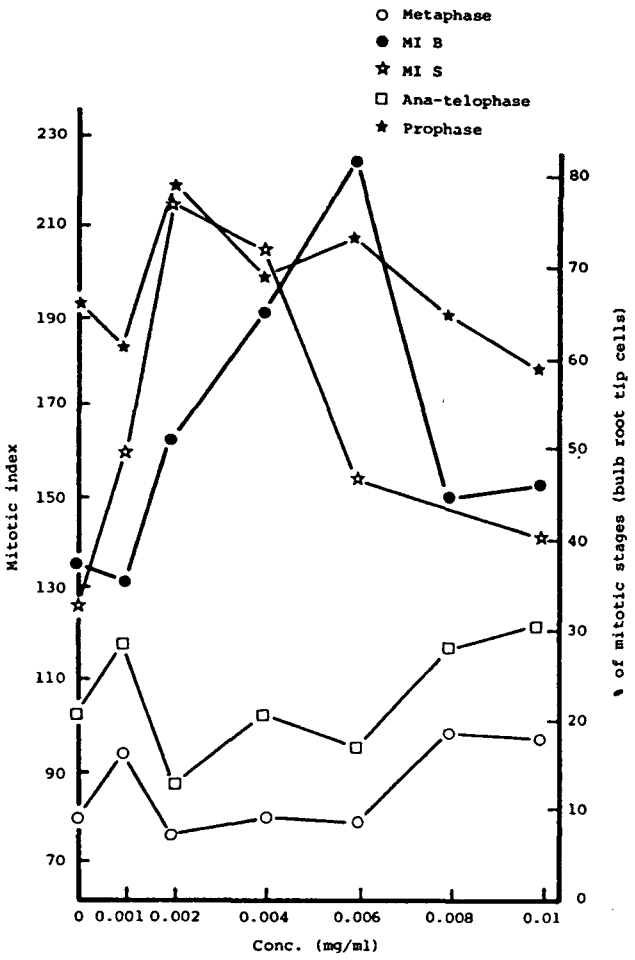

Fig. 5. Effect of different concentrations of Rg_1 on mitosis in onion bulb (MI B)and seedling (MI S) root tip cells (24-hour treatment).

Results

Concentration effect

Rg_1 stimulates mitosis in bulb and seedling root tip cells (Fig. 5). It is dose-dependent and the most effective concentrations in promoting mitosis are between 0.002–0.006 mg/ml. Rb_1 inhibits mitosis in the same type of cells (Fig. 6). The mitotic indices decrease progressively as the concentrations of Rb_1 increase. Percentage figures of different mitotic stages tell us that both ginsenosides do not block dividing cells at any particular stage (curves of proportions of different mitotic stages shown in Figures 5 and 6 represent those obtained from bulb materials).

Time course effect

During the first 18 hours of treatment, the

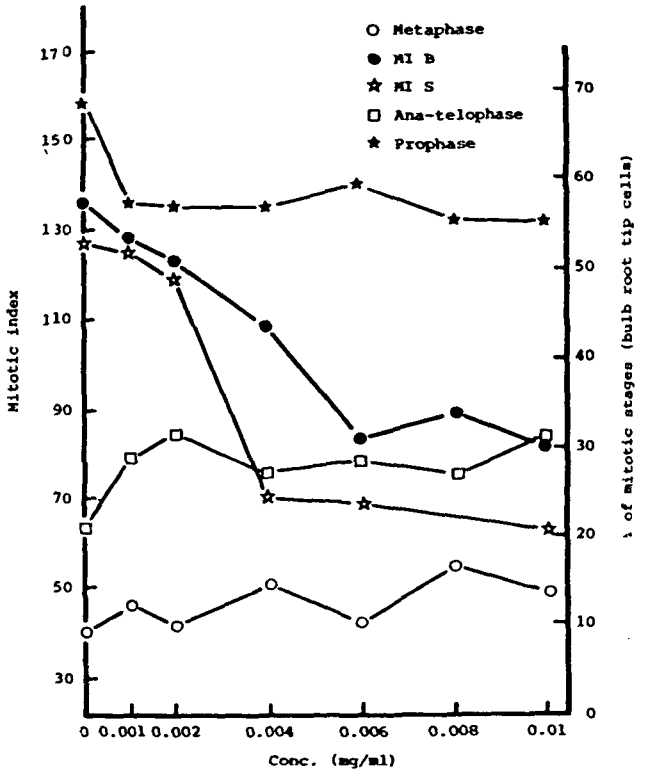

Fig. 6. Effect of different concentrations of Rb_1 *on mitosis* in onion bulb (MI B) and seedling (MI S) root tip cells (24-hour treatment).

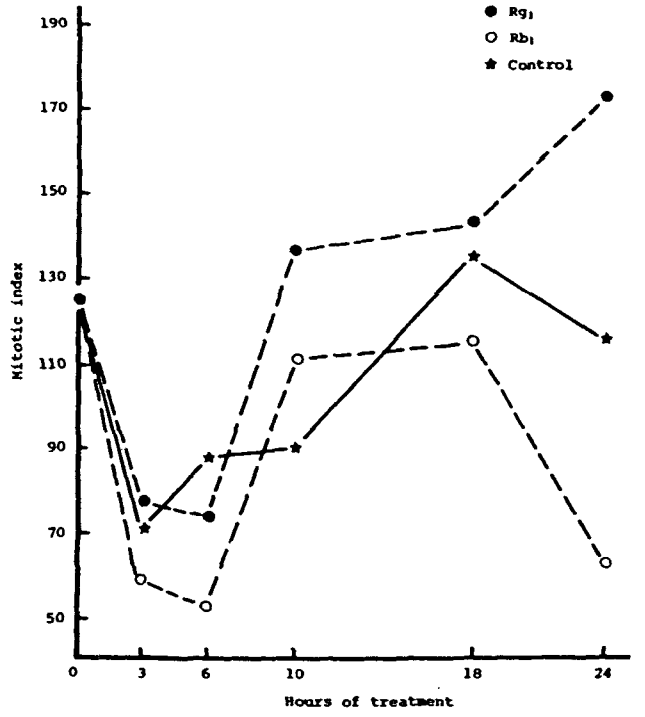

Fig. 7. Time course effect of Rg_1 and Rb_1, both at 0.004 mg/ml, on mitosis in onion seedling root tip cells.

mitotic periodicity is similar among the control, Rg_1-treated and Rb_1-treated seedling root tip cells (Fig. 7). Mitotic indices drop to a minimum at 3-or 6-hour and then rise again up to 18-hour when the effects of Rg_1 and Rb_1 on mitosis become evident. Their effects are clearly seen at 24-hour when MI is about 50% higher in Rg_1-treated cells and 46% lower in Rb_1-treated ones in comparison with that of the control. These data confirm the

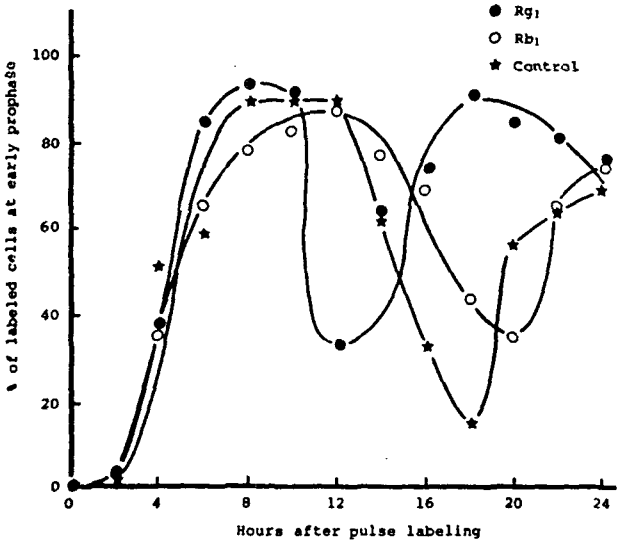

Fig. 8. Percentage of labeled cells at early prophase after various hours of pulse labeling with ^{3}H-TdR in onion bulb root tips cells.

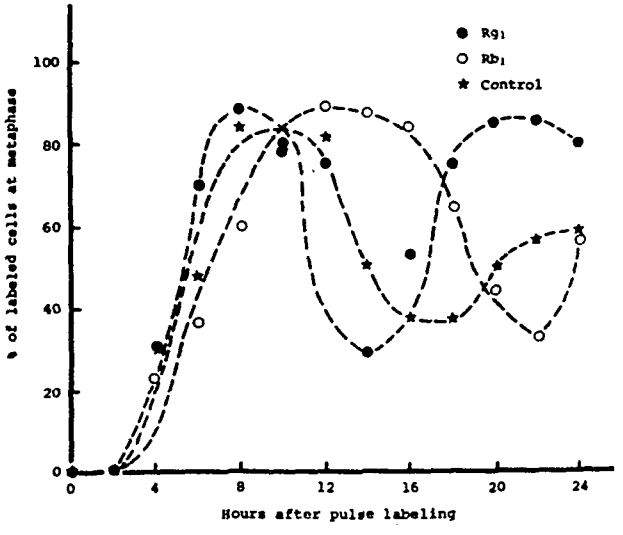

Fig. 9. Percentage of labeled cells at metaphase after various hours of pulse labeling with ^{3}H-TdR in onion bulb root tip cells.

results presented in Figures 5 and Fig 6.

Effects on durations of mitotic cell cycle and S period

Figures 8 and 9 show the variations in percentages of labeled cells at early prophase and metaphase respectively in the control, Rg_1-treated and Rb_1-treated roots. The average durations (early prophase and metaphase data) of the mitotic cycle and S period were calculated to be about 14.5 h and 8.7 h respectively for the control, 10.9 h and 5.6 h for the Rg_1-treated, and 17.0 h and 11.8 h for the Rb_1-treated roots. Thus, the mitotic cycle and S period are significantly shortened in the Rg_1-treated roots and lengthened in the Rb_1-treated ones.

Time course effect on DNA synthetic rate

The peak of DNA synthesis occurs at 6-hour in both the control and Rg_1-treated seedlings (Fig. 10). The uptake of ^{3}H-TdR is greater in the

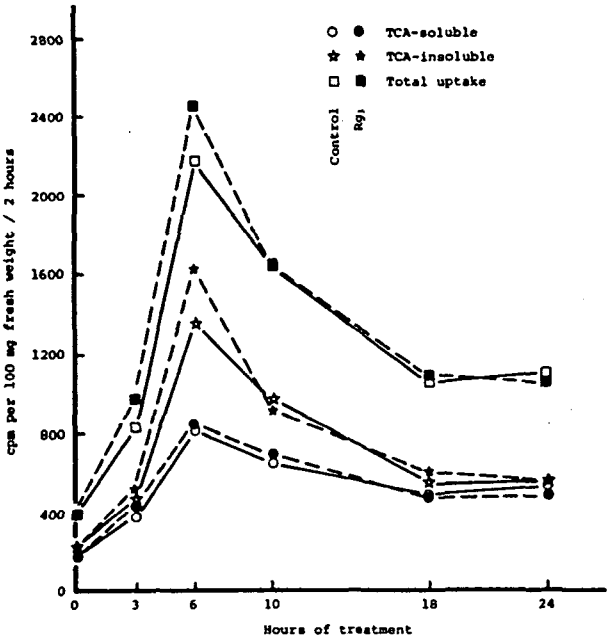

Fig. 10. Time course effect of Rg_1 (0.004 mg/ml) on the incorporation of ^{3}H-TdR in onion seedlings.

Table 4. Uptake of ^{3}H-thymidine (2 hours) per 100 mg fresh weight of onion seedlings treated with Rg_1 (0.004 mg/ml) for 0–24 hours

Hours of treatment	Treatment	cpm			%		
		TCA-soluble	TCA-insoluble	Total	TCA-soluble	TCA-insoluble	Total
0		172	215	387	—	—	—
3	control	378	457	835	100	100	100
	Rg_1	442	530	972	116.9	116.0	116.4
6	control	825	1352	2177	100.0	100.0	100.0
	Rg_1	840	1621	2461	101.8	119.9	113.1
10	control	647	981	1628	100.0	100.0	100.0
	Rg_1	699	930	1629	108.0	94.8	100.1
18	control	491	555	1046	100.0	100.0	100.0
	Rg_1	476	615	1091	97.0	110.8	104.3
24	control	545	559	1104	100.0	100.0	100.0
	Rg_1	497	559	1056	91.2	100.0	95.7

Rg_1-treated material than in the control one at 3- and 6-hour (Table 4). Similar values of uptake are found at the remaining hours.

In the Rb_1 treatment, the peak of DNA synthesis in the control seedlings also occurs at 6-hour, but in the treated ones, the high rate of DNA synthesis maintains from 10-hour to 18-hour (Fig. 11). The uptake of ^{3}H-TdR in the Rb_1-treated seedlings is greater than in the control ones at all hours except at 6 (Table 5). Thus Rb_1 delays and lengthens the peak of DNA synthesis from 6-hour to 10–18-hour.

Conclusion

1. The effects of ginsenosides Rg_1 and Rb_1 of *Panax ginseng* on mitosis in cultured human blood lymphocytes and in bulb and seedling root tip cells of onion (*Allium cepa*) were investigated.
2. Rg_1 promotes mitosis in human lymphocytes activated by phytohemagglutinin or concanagalin A and in onion root tip cells. The most effective concentrations in promoting

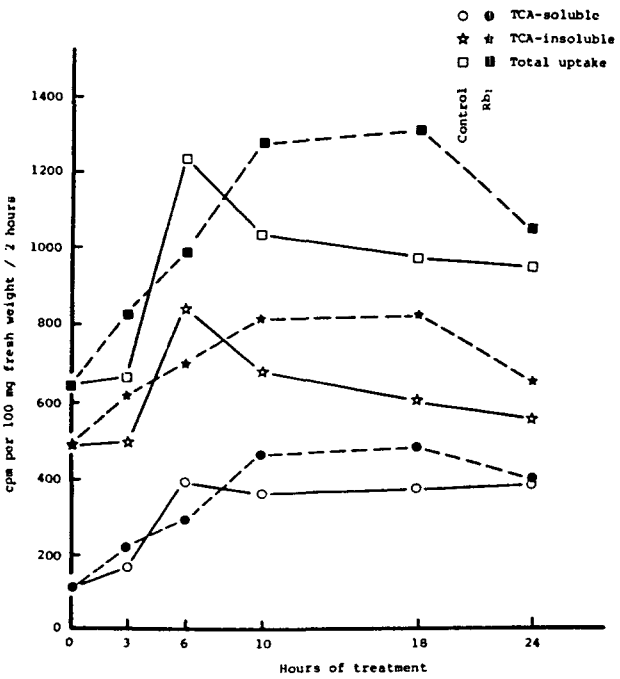

Fig. 11. Time course effect of Rb_1 (0.004 mg/ml) on the incorporation of ^{3}H-TdR in onion seedlings.

mitosis are around 0.0003–0.0005 mg/ml in the former and 0.002–0.006 mg/ml in the latter.

3. In the absence of a mitogenic lectin, Rg_1 cannot restart the quinscent human lymphocytes to divide *in vitro*. Thus it is not mitogenic.
4. Rg_1 enhances DNA synthesis in activated human lymphocytes and in onion seedlings. Experimental results indicate that it shortens DNA synthetic (S) period and mitotic cell cycle in onion bulb root tip cells.
5. Rb_1 inhibits mitosis in onion bulb and seedling root tip cells. The mitotic indices decrease progressively as the concentrations of Rb_1 increase. Results of our preliminary study indicate that Rb_1 also inhibits mitosis in activated human lymphocytes.
6. Rb_1 lengthens the S period and mitotic cell cycle in onion bulb root tip cells. It delays and prolongs the peak hour of DNA synthesis in onion seedlings.
7. Both Rg_1 and Rb_1 do not arrest the mitotic cells at any particular stage.
8. Thus Rg_1 and Rb_1 have opposite effects on mitosis in both the activated human blood lymphocytes and onion root tip cells.

Table 5. Uptake of ^{3}H-thymidine (2 hours) per 100 mg fresh weight of onion seedlings treated with Rb_1 (0.004mg/ml) for 0–24 hours

Hours of treatment	Treatment	cpm			%		
		TCA-soluble	TCA-insoluble	Total	TCA-soluble	TCA-insoluble	Total
0		153	490	643	—	—	—
3	control	164	500	664	100.0	100.0	100.0
	Rb_1	215	614	829	131.1	122.8	124.9
6	control	395	848	1243	100.0	100.0	100.0
	Rb_1	292	703	995	73.9	82.9	80.1
10	control	362	680	1042	100.0	100.0	100.0
	Rb_1	464	815	1279	128.2	119.9	122.7
18	control	377	602	979	100.0	100.0	100.0
	Rb_1	488	826	1314	129.4	137.2	134.2
24	control	382	566	948	100.0	100.0	100.0
	Rb_1	400	655	1055	104.7	115.7	111.3

Acknowledgements

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Chang: I can speak Chinese. I enjoyed your data very much and I just wonder how do you treat your seedling. How do you use the ginseng component to treat seedling? You use solution to spray on the seedling? The whole seedling or feed the root system? How do you do it?

Chao: For the unused lots of bulb, we just dip about lots into the solution, water solution. For the seedling we just treat for seedlings of use the solution.

Chang: Did you spray on it or to make a drops on some place? Did you spray the solution to the whole seedling?

Chao: Yeah, whole seedling. It's very small seedling with roots about 1 cm long.

Fulder: I must say that it was very exciting paper. I just want to make a comment that you have two possibilities for the facts on DNA synthesis, either the actual of DNA is effected or the number of start point, the initiation point is effected. And I wonder whether you thought of using technical DNA fiber autoradiography because this will distinguish between the two possibilities you have and give you some idea of the mechanism of the effect on the DNA synthesis.

Chao: We haven't used that technique yet. I think it's a good suggestion.

K. T. Choi (from Korea): Cell cycle is generally three stages Gl stage, S stage, G2 stages. I know that the time from G1 to G2 stages is different according to the kind of plant. So, I'd like to ask you question. How long does it take from G1 stage to G2 stage in your experiment?

Chao: We didn't count, because it is tedious work. From our data G1, G2 has approximately arrange between 5.2 and 5.8 hours. This is in agreement with other report.

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