Effects of *Panax Ginseng* on the Development of Morphine Tolerance and Dependence

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Abstract: The present study was undertaken to determine the inhibitory effects of orally administered ginseng saponins(GS), protopanaxadiol saponins(PD) and protopanaxatriol saponins(PT) on the development of morphine induced tolerance and physical dependence in mice, and to determine the increases in the loss of morphine tolerance and dependence. The study also sought to determine the hepatic glutathione contents which are closely related to the degree of detoxication of morphinone, a novel metabolite of morphine, and the effects of ginseng saponins on morphine 6-dehydrogenase.

The results of the present study showed that GS, PD and PT administered orally inhibited the development of morphine induced tolerance and dependence. GS, PD and PT, however, increased the loss of morphine tolerance and dependence. GS, PD and PT inhibited the reduction of hepatic glutathione concentration in mice treated chronically with morphine, and the activity of morphine 6-dehydrogenase.

So we hypothesized that these results were partially due to the dual action of the test drugs, the inhibition of morphinone production and the activation in morphinone-glutathione conjugation due to the increased glutathione level for detoxication.

Introduction

The analgesic action of morphine is very remarkable, but repeated treatment with morphine produces physical dependence, characterized by withdrawal symptoms and the development of a tolerance to most of its effects. So there must be a continuous search for morphine-type compounds which are devoid of addiction liability and are orally effective narcotic antagonists with minimum secondary effects.

A folk medicine composed of seven herbal drugs including *Panax ginseng* has been used as an antidote in the treatment of morphine-dependent patients. It was reported that its effective component was keratin of *Manis squama*[1] And my research group has reported the inhibition of the development of morphine induced tolerance and dependence in ginseng butanol fraction[2], protopanaxadiol fraction and protopanaxatriol fraction administered intraperitoneally[3], and the inhibition of the development of morphine induced dopamine receptor supersensitivity[4]. But there have been no reports that discussed the effects of ginseng saponins administered orally to morphine treated mice.

The present study was undertaken to determine the inhibitory effects of orally administered ginseng saponins(GS), protopanaxadiol saponins(PD) and protopanaxatriol saponins(PT) on the development of morphine induced tolerance and physical dependence in mice, and to determine the increases in the loss of morphine tolerance and dependence. Another purpose was to determine the hepatic glutathione contents which are closely related to the degree of detoxication of morphinone, a novel metabolite of morphine, and the effects of ginseng saponins on morphine 6-dehydrogenase.

Materials and Methods

White ICR male weighing 18-22g, in a group of 10-15, were used in all experiments. GS, PD and PT(kindly supplied from the Korea Ginseng & Tobacco Research Institute) dissolved in distilled water were administered to mice orally once a day 30 minutes prior to the injection of morphine for the tests of the effects of ginseng saponins on the development of morphine tolerance and dependence or intraperitoneally once a day for the test of morphine tolerance and dependence losses.

Measurement of analgesic tolerance

To induce morphine tolerance and dependence in mice, morphine hydrochloride (Dae-Won Pharm. Co.) 10 mg/kg was administered subcutaneously to mice every 24 hours for a period of 6 days by Kaneto's method[5].

The inhibitory degree of morphine tolerance development of the test drugs by oral administration was evidenced by the increase in analgesic response to morphine hydrochloride(5 mg/kg) as an analgesic response, estimated at 0, 30, 60 and 90 minutes by the tail flick method[6] 24 hours after the final injection of morphine and calculated as area under the curve by Kaneto and his co-workers's method[5]. The tail flick latencies to thermal stimulation were determined in seconds prior to and at 0, 30, 60 and 90 minutes after the morphine injection. A value of 10 seconds was used as a cut-off point to avoid damage to the tail. The analgesic response for each mouse was calculated by the following formula;

$$\text{Percent Analgesia}(\%) = \frac{Tt - To}{Tc - To} \times 100$$

where To is the base line or pre-morphine tail flick reaction time, Tt is the reaction time at t minutes after morphine injection, Tc is cut-off time. The base lines of tail flick latencies in different groups were around 2 ± 0.2 seconds. The effects were calculated as area under the curve(A.U.C.) that was obtained by plotting the analgesic percent on the ordinate and the time intervals(min.) on the obscissa, and expressed as a percent of the effects obtained in control animals treated only with morphine(5 mg/kg).

Measurement of inhibition of naloxone induced withdrawal

The inhibition of naloxone induced withdrawal syndrome in mice treated with morphine alone and in morphine treated mice with test drugs was estimated by the decreased scores of the withdrawal induced by naloxone(1 mg/kg, I.P.) for 30 minutes, 24 hours after the final injection of morphine on the 7th day. The abstinence syndrome was quantified by placing animals on a diaphanous circular cylinder 35 cm in diameter and 70 cm in height and by scoring the withdrawal induced by naloxone as follows; jumping and diarrhea 2 points, wetdog shake, defecation, writhing syndrome, rearing, grooming and ptosis 1 point by all or none response by the modified Tagashira and Dewey's method[7].

Induction of morphine tolerance and dependence

Morphine hydrochloride 40 mg/kg was injected s.c. to mice every 8 hours for a period of 6 days for the preparation of morphine tolerant and dependent mice[8]. The morphine tolerant and dependent mice were divided into a group of 10-15 for the tests of morphine tolerance and dependence losses.

Measurement of analgesic tolerance loss

The degree of lost morphine tolerance by the administration of ginseng saponins was evidenced by the increase in analgesic response to morphine hydrochloride 10 mg/kg s.c. by the same tail flick method as that used in the measurement of analgesic tolerance on the 7th day, 24 hours after the daily treatment in morphine tolerant mice with 100 my/kg of each saponin(i.p.) for 6 days.

Measurement of acute inhibition of dependence(naloxone induced jumping response)

The acute inhibition of naloxone induced withdrawal signs in morphine dependent mice by GS, PD and PT was estimated by the decreased number of naloxone(4 mg/kg, I.P.) induced withdrawal jumping mice for 30 minutes, 8 hours after the final injection of morphine.

The abstinence syndrome was quantified by placing animals on a diaphanous circular cylinder 35 cm in diameter and 70 cm in height and counting the number of jumping animals within 30 minutes after the pretreatment with each dose of saponins 30 minutes prior to the naloxone test[8].

Measurement of chronic dependence loss

24 hours after the morphine dependent mice were daily treated with 100 mg/kg (i.p.) of GS, PD and PT for 7 days, the degree of lost morphine dependence was estimated on the 8th day, by scoring the naloxone(1 mg/kg, i.p.) induced withdrawal signs in morphine dependent mice for 30 minutes and expressed as a percent of the control group. Scoring was made as that used in the measurement of inhibition of naloxone induced withdrawal[7].

Measurement of the hepatic glutathione contents in mice

The mice treated with morphine only and the mice treated with the test drugs regularly for 6 days were killed by decapitation on the 7th day, 24 hours after the final injection of morphine. Their livers were removed immediately and the glutathione concentration was determined by the method of Ellman as follows[9]; The removed liver were homogenized in 4 volumes of ice-cold phosphate buffer, pH 7.4, to give a suspension equivalent to 250 mg/ml of wet liver. For estimation of reduced glutathione, an aliquot was deproteinized by addition of an equal volume of 4%-trichloroacetic acid containing 1 mM Na-EDTA and after centrifugation(2000×g, 5 min), 0.5 ml of the supernatent was added to 4.5 ml of 5, 5′-dithiobis(2-nitrobenzoic acid). After mixing, absorbance at 412 nm was recorded against a reagent blank to determine the glutathione concentration. All the operations were carried out at 2-4℃.

Measurement of the inhibitory effects of morphine 6-dehydrogenase

Morphine 6-dehydrogenase was prepared by Yamano *et al's* method[10] and all operations were carried out at 2-4℃. Distilled deionized water was used to prepare all the solutions. The standard buffer was $NaH_2PO_4 \cdot Na_2HPO_4$ $\cdot$pH 8.0, containing 100 mM 2-mercaptoethanol. Centrifugation was performed at 12,000×g for 10 min. unless stated otherwise. Livers from adult male Hartlry guinea pig(600-800g) were homogenized in two volumes of 100 mM 2-mercaptoethanol and centrifuged successively at 9,000×g for 20 minutes and 105,000×g for 60 minutes.

The supernatant was treated with saturated ammonium sulfate adjusted to pH 7.4 with 25% NH_4OH to yield a fraction which precipitated between 0.45 and 0.80 saturation. The precipitate was dialyzed overnight against three changes of 100 volumes of standard buffer and insoluble materials were removed by centrifugation. The dialysate was applied to a column of Matrex green A(2.6×26 cm), previously equilibrated with 50 mM standard buffer. The column was washed with 50 mM standard buffer untill the absorbance of eluate at 280 nm decreased to less than 0.2. The enzyme was eluted with a linear gradient formed by adding 250 ml each of 50 mM standard buffer and 50 mM standard buffer containing 1 M NaCl.

Fractions of 10 ml were collected at a flow rate of 15 ml/h and were monitored for absorbance at 280 nm and enzyme activity. The active fractions were combined and concentrated by ultrafiltration with a Diaflo membrane PM-10(Amicon Corp.) and insoluble materials were removed by centrifugation.

The concentrated solution was applied to a column of Sephadex G-100(2.6×90 cm), previously equilibrated with 50 mM standard buffer and eluted with the same buffer. Fractions of 5 ml were collected at a flow rate of 15 ml/h. The active fractions were combined and concentrated. The concentrated solution was dialyzed overnight against two changes of 100 volumes of 5 mM standard buffer and insoluble materials were removed by centrifugation. The dialysate was applied to a column of DE 32(1.6×90 cm), previously equilibrated with 5 mM standard buffer and eluted with increasing ionic concentration. A linear gradient was formed by

adding 300 ml each of 5 mM and 5 ml/h. The active fractions were pooled and used for the inhibitory tests of ginseng saponins on morphine 6-dehydrogenase.

The inhibitory activity of morphine 6-dehydrogenase was measured by the change in absorbance employing 1 cm light path at 340 nm in a Shimadzu UV 150 spectrophotometer at 25℃[10]. The standard incubation mixture contained 1.5μmol. of morphine, 1.5μmol. NAD or NADP, 0.1 ml of enzyme preparation and 100 mM buffer in a total volume of 1.5 ml. As the buffer, glycine-NaOH buffer, pH 9.7, was used for NAD, and Tricine-NaOH buffer, pH 9.1, was used for NADP.

The enzyme was preincubated with the respective concentrations of each ginseng saponin at 25℃ for 2 minutes and then the reaction was initiated by the addition of morphine. For each assay, a control without substrate was performed.

Statistics

The data were expressed as mean of changes± S.E. The differences in the means for different responses in different treatment groups were analyzed by the student's t test.

Results

Inhibition of analgesic tolerance development

The base line of each group in analgesia changes was determined to check the residual effects of ginseng saponins and morphine 30 minutes prior to the tolerance test. There were no differences in the base line tail flick latencies in the different groups.

The analgesia of each group calculated as the AUC to morphine 5 mg/kg showed 3.1 in GS 50 mg/kg, 8.5 in GS 200 mg/kg, 4.9 in PD 200 mg/kg, 6.2 in PT 50 mg/kg and 8 times in PT 200 mg/kg, compared with that of the morphine control group, but no significant difference was observed in PD 50 mg/kg(Fig.1).

Inhibition of withdrawal induced by naloxone

The inhibitory degrees of naloxone induced withdrawal scores were 30% in GS 50 mg/kg 40% in GS 200 mg/kg, 23% in PD 50 mg/kg, 32% in PD 200 mg/kg, 35% in PT 50 mg/kg and 40% in PT 200 mg/kg, compared with that of the morphine control group(Fig.2).

Analgesic tolerance loss

The AUC value of tolerance loss in the morphine tolerant control group was 28.41±7.47 (n=10) on the 7th day 152 hours after the final injection of morphine. The significant losses of analgesic tolerance(recovery of analgesia) appeared in all of GS, PD and PT treated groups as shown 3.8 times in GS 100 mg/kg, 4.7

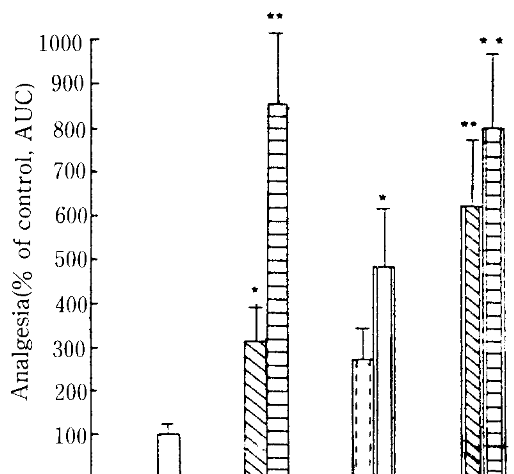
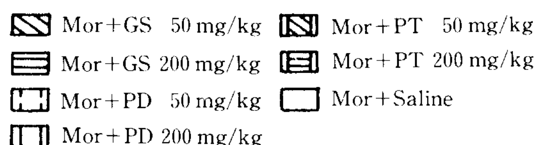


Fig.1. *Effects of GS, PD and PT administered orally on tolerance to the analgesic action of morphine in mice. Morphine 10 mg/kg was injected into the mice every 24 hours for 6 days and saline or daily doses, 50 and 200 mg/kg of GS, PD and PT were administered to the respective group. The inhibitory degree of tolerance development by GS, PD and PT was evidenced by the increase in analgesic response to morphine hydrochloride 5 mg/kg S.C.*

$*p<0.05$ $**p<0.01$



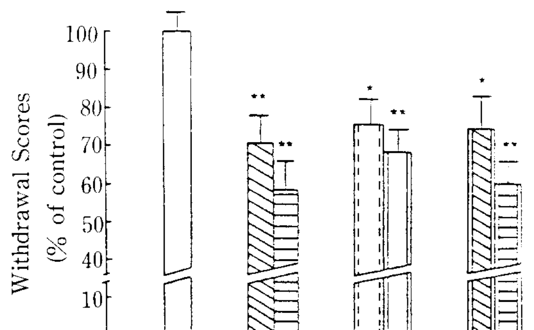
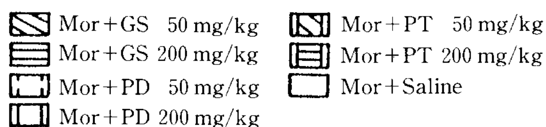


Fig.2. *Effects of GS, PD and PT on the development of morphine dependence in mice by the naloxone induced withdrawal syndrome. Each group of mice was injected with morphine hydrochloride 10 mg/kg s.c. at 24 hours intervals and administered orally with 50 and 200 mg/kg of GS, PD and PT for the respective group at 24 hours intervals for 6 days. The withdrawal test was made 24 hours after the final injection, by challanging the naloxone 1 mg/kg i.p.*

$*p<0.05$ $**p<0.01$



times in PD 100 mg/kg on the 7th day compared with that of morphine control group(Fig.3).

Acute inhibition of dependence(naloxone induced jumping response)

The naloxone induced jumping response in morphine dependent mice showed 70%, 50% and 40% respectively by the administration of each GS, PD and PT 50 mg/kg (i.p.), compared with that of morphine control group(Fig. 4).

Chronic dependence loss

The naloxone induced withrawal signs showed around 60% or so in GS and PT 100 mg/kg treated groups but 50% in PD 100 mg/kg treated group(Fig.5).

Inhibition of hepatic glutathione concentration decrease

The hepatic glutathione levels in the groups of mice treated with ginseng saponins were slightly increased from 0.03(PD 50 mg/kg) to 0. 45mmol. /g tissue(PT 200 mg/kg), compared

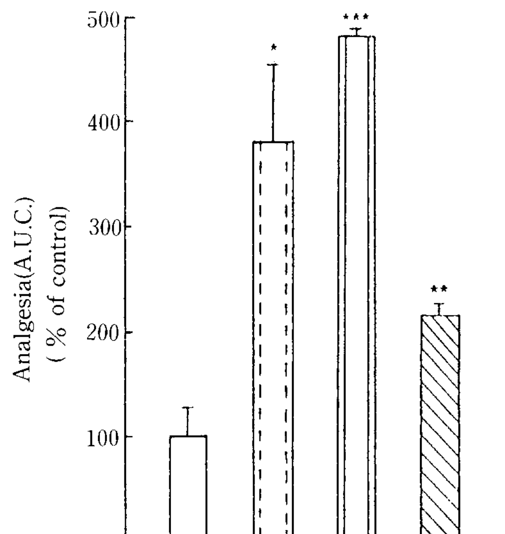
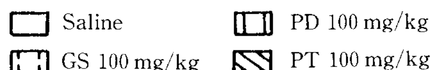


Fig.3. *Loss of analgesic tolerance in morphine tolerant mice by the administration of GS, PD and PT for 6 days. Loss of analgesic tolerance was determined 24 hours after pretreatment with daily dose, 100 mg/kg of each GS, PD and PT for 6 days. The degree of lost morphine tolerance by GS, PD and PT was evidenced by the increase in analgesic response to morphine hydrochloride (10 mg/kg s.c.) as estimated by the tail flick method and calculated at the AUC method as described in the text. Each group of mice was toleranced with morphine hydrochloride 40 mg/kg s.c. at 8 hours intervals for 6 days before the test started. The values are the means of 10 experiments.*

$*p<0.05$ $**p<0.01$ $***p<0.001$



with 3.39±0.15 μ mol./g tissue of the saline group. However, the glutathione level in the morphine control group was decreased to 2.41± 0.12 μ mol./g tissue.

The glutathione levels of the groups treated with morphine and test drugs were observed from 2.77±0.09(PD 200 mg/kg) to 2.26±0.1 μ mol./g tissue(PT 200 mg/kg), showing the significant inhibitory effects in the hepatic glutathione level decreases, compared with that of the morphine control group. However no

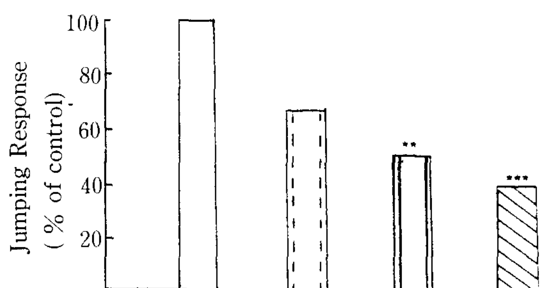


Fig.4. *Acute inhibitory effects of GS, PD and PT in morphine dependent mice by naloxone induced jumping response. The acute inhibition of naloxone induced withdrawal signs in morphine dependent mice by GS, PD and PT was estimated by the decreased number of naloxone(4 mg/kg i p.) induced withdrawal jumping mice for 30 minutes, 8 hours after the final injection of morphine, and naloxone was administered to morphine dependent mice 30 minutes after the pretreatment with each dose of ginseng saponins.*

$*p<0.05$ $**p<0.01$ $***p<0.001$

Mor+Saline; Mor+PD 50 mg/kg; Mor+GS 50 mg/kg; Mor+PT 50 mg/kg

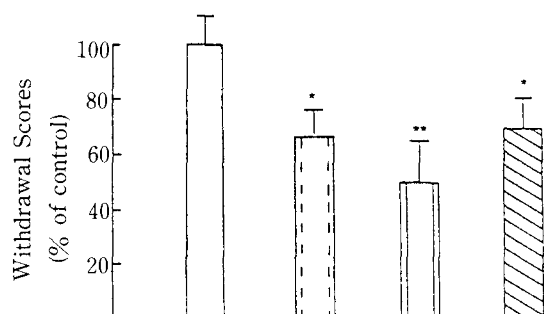


Fig. 5. *Effects of GS, PD and PT on the chronic loss of dependence in morphine dependent mice by the scoring method. The degree of lost morphine dependence by GS, PD and PT was evidenced by the decreased percents in withdrawal response to naloxone hydrochloride 1 mg/kg i.p. as described in the text. Daily dose, 100 mg/kg of each GS, PD and PT was pretreated to the respective group for 7 days and 24 hours after, the measurement was made. The values are the means of 7 experiments.*

$*p<0.05$ $*p<0.01$ $***p<0.001$

MOR+Saline; Mor+PD 100 mg/kg; Mor+GS 100 mg/kg; Mor+PT 100 mg/kg

significant difference was shown in the group treated with GS 50 mg/kg or PD 50 mg/kg(Fig. 6).

Inhibitory effects of morphine 6-dehydrogenase

The enzyme was inhibited about 50% by 0.01% of PT (corresponding to approximately 0.125 mM based on a average M.W. 800) at the physiological condition(pH 7.4). PT is a little bit more effective than naloxone. But also the enzyme was inhibited about 18.1% and 15.8% respectively by 0.1% of each GS and PD. So, PT functioned as a bioactive inhibitor(Table I).

Discussion

Kim and his co-workers reported that GS, PD and PT administered intraperitoneally inhibited the development of morphine induced tolerance and physical dependence[11] and their active components were Rb1 and Rg1[12]. In this experiment, GS, PD and PT administered orally were also found to inhibit the development of morphine induced tolerance and physical dependence, while they increased the loss of morphine tolerance and dependence. In the liver of the mice, a portion of morphine was metabolized into morphinone which was a novel metabolite of morphine and had 9 times the toxicity and 0.5 time the analgesic activity of morphine based on LD_{50} and ED_{50} value each in mouse (*s.c.*). The present research showed that morphine 6-dehydrogenase which catalyzed morphinone production from morphine was inhibited by ginseng saponins, especially PT, *in vitro*.

An aliquot of morphinone conjugated with glutathione was closely related to the detoxication process and the other aliquot of morphinone was metabolized into morphinone-protein SH conjugate concerned with the devel-

Table I. *Effects of ginseng saponins, protopanaxadiol saponins and protopanaxatriol saponins on guinea pig liver morphine 6-dehydrogenase. The inhibitory effects were measured as described in the text*

Saponin fraction	Concentration(%)	Inhibition (%)			
		with NADP		with NAD	
		at optimum pH	at pH 7.4	at optimum pH	at pH 7.4
	1.0	36.6	–	–	–
Ginseng saponins	0.1	7.9	18.1	9.5	9.1
	0.01	0	3.6	0	0
	0.001	0	0	0	–
	1.0	44.9	–	–	–
Protopanaxadiol saponins	0.1	7.3	15.5	6.9	9.1
	0.01	0	0	0	0
	0.001	0	0	0	0
	1.0	75.1	–	–	–
Protopanaxatriol saponins	0.1	53.1	67.8	44.4	67.3
	0.01	34.5	51.1	28.6	45.4
	0.001	4.0	11.9	4.8	–
	0.0001	0	3.9	0	–

-, not determined

opment of morphine induced tolerance and physical dependence by covalent binding to the sulfhydryl group of opiate receptor(Fig.7) [13]. Schole *et al* reported that the standardized ginseng extract G 115 significantly increased the glutathione level of rat liver within minutes[14], as observed by similar increase of glutathione level in ginseng saponins treated mice(Fig.6).

The inhibition of hepatic glutathione level decrease in morphine treated mice with GS, PD and PT showed the inhibitory tendency of the development of morphine induced tolerance and physical dependence in this experiment. So we hypothesized that these results were partially due to the dual action of the test drugs, the inhibition of morphinone production and the activation in morphinone-glutathione conjugation due to the increased glutathione level for detoxication(Fig.7). In addition, the newly equilibrated state of neurogic function rather than the changed brain levels of neurotransmitters on the inhibition of morphine tolerance and dependence development by ginseng saponins can also be considered as acting points, as Takahashi and Kaneto discovered the newly equilibrated state of adrenergic function as well as the inhibition of the development of morphine tolerance in mice treated daily with a small dose of reserpine[15].

The mechanism of inhibition of abstinence syndrome in morphine dependent-tolerant animals by ginseng saponins remains unclear. Several neurotransmitters, Ach, D.A. and c-AMP, have been implicated in the abstinence syndrome. The expression of abstinence syndrome is associated with an increase in brain D. A. level[16], an increase in c-AMP level[17] and a decrease in brain Ach level[18]. The studies involving the effects of ginseng saponins on whole brain neurotransmitter levels and on the neurotransmitters turn-over rates in the total brain and in the various regions of brain have yielded conflicting data. Most of the studies showed increases in noradrenaline, dopamine, serotonin and c-AMP in ginseng saponin treated animals[19,20] while there was no report on Ach level. Norepinephrinergic neurons in the brain

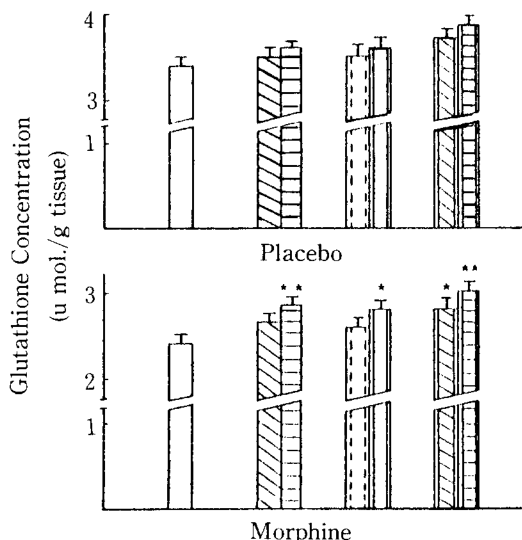
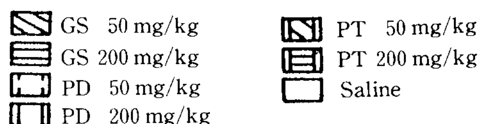


Fig. 6. *Effects of GS, PD and PT on the inhibition of hepatic glutathione level decrease in mice on 7th day. Morphine 10 mg/kg was injected into the mice every 24 hours for 6 days, and 50, 200 mg/kg of GS, PD and PT were administered orally to the respective group once a day 30 minutes prior to the morphine injection for 6 days.*

$*p<0.05$ $**p<0.01$ $***p<0.001$



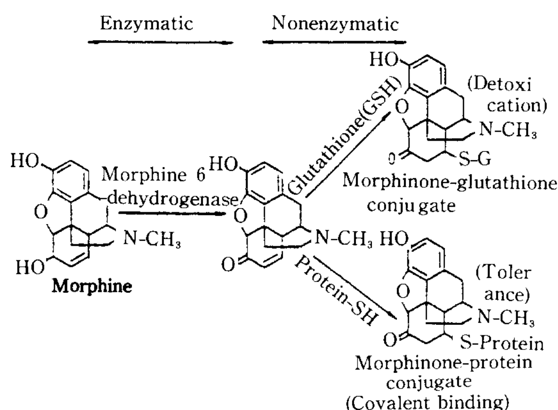


Fig. 7. *Metabolisms of morphine to morphinone and its conjugates.*

of the mice were more influenced by oral chronic treatment of *panax ginseng* than the dopaminergic neurons[21]. We hypothesized that the inhibitory effects of morphine induced physical dependence by GS, PD and PT were closely related to the changed ratios of EP, NE, DA and serotonin as well as the newly equilibrated state of neurogic function in brain.

The results of the present study showed that GS, PD and PT administered orally inhibited the development of morphine induced tolerance and physical dependence, while they increased the loss of morphine tolerance and dependence, and also inhibited the reduction of hepatic glutathione concentration in mice treated chronically with morphine and the activity of morphine 6-dehydrogenase.

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