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Antinociceptive pattern of flavone and its mechanism as tested by formalin assay

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Flavone, dextrose and long swim stress exhibited antinociception. Degree of antinociception was greater with long swim stress as compared to flavone or dextrose. Combination of these treatments resulted in potentiation of antinociception. Naloxone (opioid antagonist; 5 mg/kg ip) antagonised flavone or long stress induced antinociception showing opioid mediated mechanism, however, failed to reverse the potentiated antinociceptive component recorded in long stressed animals which received flavone and dextrose. Antinociceptive activity of flavone, dextrose and long swim stress which was documented by acetic acid assay has been confirmed in the present study. Role for opioid system in this action has been demonstrated. Therefore, formalin test can also be considered as an useful assay procedure for testing flavonoids. However, like acetic acid assay this assay procedure also has the limitation that it is unable to detect minor changes in the degree of antinociception produced by physiological interventions such as long swim and dextrose.

Assessment of antinociception in experimental animals is of great concern to the scientists working in the field of pain. Despite the availability of many procedures the choice is becoming difficult. The notion that for testing opioid like analgesics, thermal assay procedure may be ideal and for aspirin type analgesics chemical assay be suitable, is changing. Hyashi and Takemori[1] have reported that ED_{50} of morphine is much lower (0.3 mg/kg) in chemical assay in contrast to thermal assay (10 mg/kg). They have advocated this assay procedure as sensitive with minimal nociception. Though this procedure is widely accepted, debate is on regarding the validity of this method especially where chronic pain is to be measured. Besides, the choice of assay procedure was found to be an important factor in order to obtain uniform results while investigating the mechanisms involved in the antinociception elicited by various agents[2]. Subsequently, antinociception was also attained by subjecting animals to various stressful conditions such as electric foot shock, restraint, swimming in water, etc.[3-12]. Recently, it has been described that in a same test procedure, an acute as well as chronic pain could be effectively measured. This test involves injection of formalin in the hind paw of the rats[13]. This formalin test has been later extended to mice and reported to be a useful tool for evaluating mild analgesics[14]. In the recent past, formalin test method has been employed for studying the antinociceptive response in diabetic animals since a chronic pain (similar to clinical situation) assessment is feasible with this method[15-18].

Flavonoids have been documented to exhibit anti-inflammatory, antiulcer and antinociceptive properties in acetic acid induced abdominal constriction procedure[19,20]. Recently, a study investigating the possible utility of 7-hydroxy flavone in management of painful diabetic neuropathy provided promising results as assessed by acetic acid assay[21]. Since this aspect involves changes in blood glucose level and chronic pain, a study employing the formalin assay procedure with flavonoids was considered worthwhile. Therefore, in the present study the antinociceptive action of flavone, a basic nucleus of flavonoids, was studied using formalin test. The experimental protocol was designed in such a way that physiological changes in blood glucose (hypo and hyperglycemia) were achieved. Effect of such changes *per se* as well as on flavone induced antinociception was recorded. Additionally, the possibility of involvement of opioid system in the antinociceptive response measured by this procedure was also investigated.

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Table 1—Antinociceptive response of flavone, dextrose, long stress alone or in combination and antagonistic effect of naloxone [Values are mean ± SE]

Treatments	n	Licking / biting response in seconds	
		Acute phase (0-10 min)	Chronic phase (10-30 min)
Vehicle	12	49.3 ± 3.3	80.1 ± 9.3
Flavone	6	19.3 ± 3.7[a]	8.7 ± 2.4[a]
Dextrose	6	18.7 ± 3.0[a]	17.3 ± 1.8[a]
Long stress(3 min swim)	7	3.1 ± 2.0[a]	0[a]
Flavone + Dextrose	6	9.7 ± 1.4[b]	1.0 ± 0.7[c]
Flavone + Long stress	6	0	0
Dextrose + Long stress	6	0.7 ± 0.4	0
Flavone + Dextrose + Long stress	6	0	0
Flavone + Naloxone	6	66.7 ± 3.0[g]	1.1 ± 0.6[f]
Naloxone + Long stress	6	17.7 ± 2.6[h]	0
Flavone + Naloxone + Long stress	6	0	0
Flavone + Dextrose + Naloxone	6	15.8 ± 2.1[i]	0
Flavone + Dextrose + Naloxone +Long stress	6	1.0 ± 0.7	0

[a]$P < 0.001$ when compared with vehicle treatment value
[b]$P < 0.05$ and [c]$P < 0.01$ when compared with flavone treatment value
[f]$P < 0.02$ and [g]$P < 0.001$ when compared with flavone treatment value
[h]$P < 0.01$ when compared with long stress treatment value
[i]$P < 0.05$ when compared with flavone + dextrose treatment value

Swiss male albino mice (20-25 g) were used for this study. They were housed at room temperature (28°-30°C) in the animal house, with 12 hr light and dark cycle with free access to water and food (chow obtained from Gold Moghur, Bangalore). Each group consisted of 6 animals. All the experiments were carried out between 1000 and 1200 hrs to avoid variation.

Formalin test described earlier[22] was followed. Each mouse was placed in a observation chamber 5 min before the injection of diluted formalin to allow acclimatisation to the new environment. Ten µl of formaldehyde (1%) in saline was administered into the left hind paw. Each animal was then returned to the observation chamber and nociceptive response was recorded for a period of 30 min. Summation of time (sec) spent in licking and biting of formalin injected paw during each 5 min block was measured as an indicator of pain response. Duration of responses in first 10 min and that from 10 to 30 min represent acute and chronic phase respectively.

Flavone—Flavone, synthesized according to Baker[23], was gifted by M/s. Herborganics, Chennai and characterised by recording melting point and by chromatographic procedures using the authentic sample. Flavone (50 mg/kg) suspended in carboxymethyl cellulose (1%) was injected sc, 60 min prior to formalin injection. This dose was found to be an effective dose in acetic acid induced abdominal constriction model.

Swim stress—Animals were allowed to swim individually in a polypropylene container (51 × 38 × 30 cm) filled with water upto 15 cm height at room temperature (28°-30°C) for 3 min (long stress)[24], wiped with a dry towel and injected formalin in the left hind paw.

Dextrose—Dextrose (50 mg/animal) was injected ip, 15 min prior to formalin injection. The dose of dextrose was selected based on pilot studies.

Role of opioid system—Naloxone (5 mg/kg, ip), an opioid antagonist, was employed to investigate the role of opioid system in antinociceptive response of flavone as assessed by formalin test. Naloxone was administered either 50 min after flavone administration or 10 min prior to swim stress.

The data was analysed statistically by ANOVA followed by Dunnet's *t* test.

The vehicle treated animals spent approximately 50 sec in the acute phase and 80 sec in the chronic phase of formalin induced nociception. Flavone treatment significantly reduced the time spent by the animal in the acute as well as chronic phase (Table 1). Degree of reduction was more in chronic phase (89.1%) as compared to acute phase (60.9%). Similarly, admini-

stration of dextrose reduced the time spent in licking in both acute and chronic phases. In contrast to flavone, degree of reduction was almost comparable. Long stress (swimming in water for 3 min) markedly reduced the reaction time in acute phase and completely abolished the biting response in chronic phase. From the above results, it is clear that degree of antinociception induced by various manoeuvers followed in this study can be graded as long stress > flavone > dextrose. When dextrose was administered in flavone pretreated animals (45 min after flavone), degree of inhibition of responses in both phases were markedly enhanced. When flavone pretreated animals were subjected to long stress, the licking and biting response was absent in both acute and chronic phases. Similarly, when dextrose pretreated animals were subjected to long stress mild biting response was observed that too only in acute phase. Finally, when flavone and dextrose pretreated animals were allowed to swim in water, formalin induced licking and biting responses were not observed (Table 1).

Naloxone (5 mg/kg, ip) treatment in flavone pretreated animals antagonised the inhibitory response of flavone on the time spent in formalin induced licking and biting response. In fact, the animal spent more time in licking as compared to vehicle pretreated animals. In contrast, the time spent in chronic phase was further reduced indicating an enhanced antinociceptive response (Table 1).

Naloxone pretreatment attenuated the antinociceptive response elicited by the animal in acute phase, but failed to modify the long stress induced responses during chronic phase (Table 1).

Treatment with naloxone in animals which received flavone and dextrose partially enhanced the time spent in early phase when compared with flavone and dextrose treated animals in acute phase without altering chronic phase response. In contrast, when the animals that received flavone, dextrose and naloxone were subjected to long stress no significant change in the time spent in licking/biting response either with acute or chronic phase was recorded as compared with those recorded in animals which did not receive naloxone (Table 1).

In conformity with earlier report on antinociceptive activity of flavone tested by acetic acid induced abdominal constriction assay procedure[25], in the present study, flavone showed significant antinociceptive response in the acute phase (60.9%) and a marked response (89.1%) in the chronic phase. Acute phase of the flavone action was reversed by naloxone treatment indicating that the action was mediated through opioid mechanism. Failure by naloxone to antagonize the chronic phase, rather an enhancement, suggest that opioid pathways may not be involved in this chronic phase which possibly represents inflammatory component[13].

It has been documented that exogenous administration of dextrose (hyperglycemic state) elicit antinociceptive response by other assay procedures[26,27]. The present findings in formalin test is in agreement with the above data. However, in contrast to flavone, not much difference in degree of antinociception was observed between acute and chronic phases. Similarly, in agreement with earlier findings[24] long swim stress (hypoglycemic state) produced almost 100% antinociception in both the phases. While the acute phase of antinociception of dextrose was attenuated by naloxone, the chronic phase remained unaltered. This observation confirms that the acute phase of antinociceptive response in formalin test utilises opioid pathways, whereas the chronic phase does not.

Enhanced antinociceptive response recorded in flavone and dextrose, flavone and long stress, dextrose and long stress and in the animals which received all the three treatments suggested that flavone, dextrose and long stress possibly utilized a similar mechanism to produce antinociceptive response. Failure by naloxone to antagonise the potentiated antinociceptive response recorded in animals which were subjected to either flavone and long stress or a combination of flavone, dextrose and long stress indicated that potentiation was independent of opioid pathways. However, a partial antagonism of potentiated acute phase of antinociception elicited by flavone and dextrose confirmed a role for opioid pathways in acute phase, possibly the long stress induced antinociception when combined with other two treatments was more profound such that this assay procedure is not sensitive enough to record the reversal by naloxone. It has been established that the long stress induced antinociception represents the opioid component and is very effective[24].

To conclude, the present study confirmed the antinociceptive effect of flavone. Involvement of opioid mechanism in combination of flavone with dextrose and long swim stress was evident especially in acute phase of formalin induced nociception. However, chronic phase of antinociception induced by these paradigms was not modified by naloxone thus indi-

cating that mechanisms other than opioid pathways may be operative during this phase. It has been reported that chronic phase of formalin nociception may result from an inflammatory response[13]. Abolition of nociceptive response observed during chronic phase of formalin induced nociception may be due to an anti-inflammatory effect of flavone.

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References

1 Hyashi G & Takemori AE, *Eur J Pharmacol*, 16 (1971) 63.
2 Sivam S P & Ho I K, *Life Sci*, 37 (1985) 199.
3 Akil H, Madeen J IV, Patrick R L & Barchas J D, in *Opiates and endogenous opioid peptides*, edited by H W Kosterlitz (Elsevier/North Holland Biomedical Press, Amsterdam) 1976, 63.
4 Bodnar R J, Kelley P D, Spiaggia A, Ehrenberg C & Glusman M, *Pharmacol Biochem Behav*, 8 (1978) 667.
5 Lewis J W, Cannon J T & Liebeskind J C, *Science*, 208 (1980) 623.
6 Izumi R, Takahashi M & Kaneto H, *JPN J Pharmacol*, 33 (1983) 1104.
7 Takahashi M, Izumi R & Kaneto H, *JPN J Pharmacol*, 35 (1984) 175.
8 Takahashi M, Tokuyama S & Kaneto H, *JPN J Pharmacol*, 44 (1987) 283.
9 Takahashi M, Tokuyama S & Kaneto H, *JPN J Pharmacol*,46 (1988) 418.
10 Takahashi M, Senda T, Tokuyama S & Kaneto H, *JPN J Pharmacol*, 53 (1990) 487.
11 Tokuyama S, Takahashi M & Kaneto H, *J Pharmacodyn*, 14 (1991a) 357.
12 Tokuyama S, Takahashi M & Kaneto H, *J Pharmacodyn*, 14 (1991b) 637.
13 Dubuisson D & Dennis S G, *Pain*, 4 (1977) 161.
14 Hunskaar S, Fasmer B & Hole K, *J Neurosci Method*, 14 (1985) 69.
15 Acton J, Mckenna J E & Melzack R, *Exp Neurol*, 117 (1992) 94.
16 Calcutt N A, Malmberg A B, Yamamoto T & Yaksh T L, *Pain*, 58 (1994) 413.
17 Takeshita N, Ohkubo Y & Yamaguchi I, *J Pharmacol Exp Ther*, 275 (1995) 23.
18 Takeshita N & Yamaguchi I, *Br J Pharmacol*, 116 (1995) 3133.
19 Parmar N S & Ghosh M N, *Indian J Pharmacol*, 10 (1978) 277.
20 Ramaswamy S, Pillai N P & Parmar N S, *Indian J Pharmacol*, 11 (1979) 135.
21 Venkataramanan P E, *A study on the role of changes in blood glucose, ATP sensitive potassium channel and GABA in 7-hydroxy flavone induced antinociception and delay in small intestinal transit*, Ph.D thesis submitted to Dr MGR Medical University, Madras, 1998.
22 Takeshita N & Yamaguchi I, *J Pharmacol Exp Ther*, 281 (1997) 315.
23 Baker W, *J Chem Soc*, (1934) 1381.
24 Tierney G, Canmody J & Jamieson D, *Pain*, 46 (1991) 89.
25 Thirugnanasambantham P, *Synthesis and structure activity study of flavone and its derivatives*, Ph.D thesis submitted to Madras University, Madras, 1987.
26 Singh I S, Challenge T K & Ghosh J J, *Eur J Pharmacol*, 90 (1983) 437.
27 Reddy P R M K, Shankar Raman R & Ramaswamy S, *Indian J Physiol Pharmacol*, 42 (1998) 131.