

## L-arginine-nitric oxide pathway modulates morphine-induced inhibition of gall bladder emptying in mice

Nitric oxide (NO) serves as a neurotransmitter in the non-adrenergic and non-cholinergic (NANC) inhibitory pathway of the gastrointestinal tract.<sup>1</sup> Several findings have demonstrated the presence of NO-synthase containing nerve fibers and neurons in the wall of the gall bladder and biliary pathways of several mammals including man<sup>2-4</sup> and NO serves as a modulator in the control of the contractions of the gall bladder and emptying.<sup>5</sup> This concept is supported by the recent findings that administration of L-arginine, the precursor of NO or NO donor, glyceryl trinitrate impairs postprandial gall bladder emptying.<sup>6,7</sup> Morphine and other opioids cause a marked increase in the pressure in the biliary tract and prevent emptying of the gall bladder, mainly due to spasm of the sphincter of Oddi.<sup>8,9</sup> Biliary stasis induced by both acute and chronic morphine treatment may produce symptoms that may vary from biliary colic to gallstone formation<sup>9,10</sup> and this effect of morphine-induced biliary stasis is naloxone reversible.<sup>9</sup> Similarly, sublingually administered nitroglycerin is employed therapeutically to decrease the elevated intra biliary pressure due to morphine.<sup>11</sup> In the present study, we have examined the effect of L-arginine, the physiological precursor of NO on morphine-inhibited gall bladder emptying in mice, in order to investigate whether endogenous NO plays a role in the biliary tract effects of morphine.

All experiments carried out were approved by the institutional animal ethics committee. Male Swiss albino mice  $20 \pm 1$ g (Vaccine institute, Belgaum, India) were divided into groups of ten each and were housed under standard animal room conditions (12 h light/ dark cycle) with free access to food and water for at least one week before the experiment. The animals were starved for 24 h before the experiment with free access to water. Drugs used were, L-arginine (Sigma, St.Louis, MO, USA), D-Arginine and N<sup>G</sup>-nitro-L-arginine (L-NNA) (Fluka, AG, Buchs SG, Switzerland), Morphine sulphate (Astra IDL, Bangalore, India), and Naloxone hydrochloride (K.L.E.S's Hospital and Medical Research Center, Belgaum, India). All the drug solutions were prepared on the day of the experimentation in 0.9% w/v saline and were administered intraperitoneally in a volume of 1ml per 100 g of body weight.

Biliary motility was determined by the method of Valsecchi & Toson G.<sup>12</sup> Animals of one group received normal saline (0.9% w/v NaCl) by intraperitoneal (0.25 ml) and oral (1 ml) routes to establish the normal weight of the gall bladder. In all other groups, gall bladder emptying was induced by oral administration of 1ml of a 30% suspension of lyophilised egg yolk in

saline, 8 min after intraperitoneal administration of drug or saline. Animals were killed by ether inhalation 15 min after oral administration of saline or egg yolk. Gall bladders were removed by sectioning the cystic duct and were weighed. The standard weight of the gall bladder was calculated for each group. Percent inhibition of the emptying was calculated as: % Inhibition of emptying =  $(T_1 - C) \times 100 / B - C$ , where B = mean weight (mg) of gall bladder in saline (*i.p.* and *p.o.*), C = mean weight (mg) of gall bladder in the egg-yolk-treated control group,  $T_1$  = mean weight of gall bladder (mg) in the drug-treated group. Initially, the inhibitory effect of morphine (1, 2, and 4 mg/kg, *i.p.*) on egg yolk-induced gall bladder emptying was measured. In other experiments, mice were pre-treated with either naloxone (2 mg/kg, *i.p.*) or L-arginine (5-15 mg/kg, *i.p.*)<sup>6</sup> or D-arginine (10 mg/kg, *i.p.*) or L-arginine (15 mg/kg, *i.p.*) plus L-NNA (10 mg/kg, *i.p.*) 20 min prior to the morphine (2 mg/kg, *i.p.*) administration to assess their effect on morphine-inhibited egg yolk-induced gall bladder emptying. In the combined treatment group L-NNA was administered 30 min prior to the L-arginine administration.

Results were expressed as mean  $\pm$  SEM. Data were analyzed by one-way ANOVA followed by Dunnett's test for dose response comparisons and by Student's 't' test for unpaired data for comparing two means. A value of  $P < 0.05$  was considered statistically significant. The results are shown in Table 1. The mean weight of the gall bladder in saline-treated (both *p.o.* and *i.p.*) mice was  $21.74 \pm 0.48$  mg. Oral administration of egg yolk resulted in emptying of the gall bladder and the average weight of the gall bladder was  $7.28 \pm 0.8$  mg. Administration of morphine (1,2,4 mg/kg) inhibited the egg yolk-induced gall bladder emptying in a dose-related manner; there was a significant ( $P < 0.001$ ) increase in the average weight of the gall bladder of these groups when compared with control. The inhibitory effect of morphine (2 mg/kg) on egg yolk-induced gall bladder emptying was blocked by pre-treatment with opioids receptor antagonist naloxone (2 mg/kg). Similarly, pre-treatment with L-arginine (5, 10, 15 mg/kg) significantly abolished the inhibitory effect of morphine (2 mg/kg) on egg yolk-induced gall bladder emptying in a dose-related manner; the average weight of the gall bladders of these groups was significantly ( $P < 0.001$ ) reduced when compared with the morphine (2 mg/kg)-treated group. Further, the inhibitory effect of morphine was completely abolished by a 15 mg/kg dose of L-arginine, since the average weight of the gall bladders of this group was not different from the control group. In con-

Table 1

**Effect of L-arginine, D-arginine, naloxone (NAL) and L-arginine plus N<sup>6</sup>-nitro-L-arginine (L-NNA) on morphine-inhibited egg yolk-induced gall bladder emptying in mice**

Treatment	Dose (mg/kg)	Gall bladder weight (mg) mean±SEM	Inhibition of emptying (%)	One-way ANOVA
Saline (Control)	0.25 ml	7.22 ± 0.8	—	
Morphine	1	14.9 ± 0.11 <sup>a</sup>	52.88	F = 116
	2	17.14 ± 0.52 <sup>a</sup>	68.31	P<0.001
	4	21.22 ± 0.51 <sup>a</sup>	96.34	df (3, 36)
Morphine	2	17.14 ± 0.52	68.31	
Morphine + L-arginine	2 + 5	15.60 ± 0.47	57.70	
Morphine + L-arginine	2 + 10	13.47 ± 0.41 <sup>b</sup>	43.03	F = 1218
Morphine + L-arginine	2 + 15	8.58 ± 0.30 <sup>b</sup>	9.53	P<0.001
Morphine + naloxone	2 + 2	7.86 ± 0.15 <sup>c</sup>	4.04	df (6, 63)
Morphine + L-arginine + L-NNA	2 + 15 + 10	16.98 ± 0.57 <sup>d</sup>	66.79	
Morphine + D-arginine	2 + 10	18.71 ± 0.36	79.12	

Drugs were given to treatment groups *i.p.* 8 min before oral administration of 1 ml of 30% suspension of lyophilised egg yolk in saline. Control animals received saline by both routes. <sup>a</sup>P < 0.01 compared with saline control, <sup>b</sup>P<0.01 compared with morphine (2 mg/kg), <sup>c</sup>P<0.01 compared with morphine (2 mg/kg), <sup>d</sup>P<0.01 compared with morphine (2 mg/kg) plus L-arginine (15 mg/kg)

trast, D-arginine (10 mg/kg) had no effect on morphine (2 mg/kg)-inhibited egg yolk-induced gall bladder emptying. Pre-treatment of animals with L-NNA (10 mg/kg) counteracted the effect of L-arginine (15 mg/kg) against morphine (2 mg/kg)-inhibited egg yolk-induced gallbladder emptying. We found that the inhibitory effect of morphine on egg yolk-induced gall bladder emptying was abolished by pretreatment with L-arginine and this effect of L-arginine was mediated through the L-arginine - NO pathway.

The presence of egg yolk in the duodenum releases CCK, which is a physiological stimulus for gall bladder emptying by inducing contraction of the gall bladder muscle and relaxing the sphincter of Oddi.<sup>12,13</sup> In the present study pre-treatment of mice with morphine inhibited egg-yolk-induced gall bladder emptying in a dose-dependent manner and the inhibition was naloxone-sensitive suggesting the suitability of this model for the objective of the present study. Morphine inhibits CCK-induced gall bladder emptying by inducing spasm of the sphincter of Oddi.<sup>10</sup> The major observation of the present study was the reversal of the inhibitory effect of morphine on egg yolk-induced gall bladder emptying by L-arginine, which was stereospecific and L-NNA sensitive, suggesting the participation of endogenous NO. Nerve fibers staining for NADPH-D a histochemical marker for neuronal NOS have been described in the mouse biliary tract.<sup>2</sup> Further, pharmacologically, NO maintains low basal smooth muscle tone of the gall bladder and reduces the contraction induced by CCK.<sup>14, 15</sup> A conceivable explanation for our results is that endogenous NO released in the biliary tract after L-arginine treatment exhibits a tonic relaxing influence on the sphincter of Oddi and the biliary tract which enables the CCK to induce emptying of the gall bladder. However, with the present study we could not demonstrate the site of action of the infused L-arginine, whether NO is exerting its relaxing effect on the gall bladder smooth muscle itself or on the sphincter of Oddi. This could be resolved by biochemical estimation of NOS activity following L-arginine administration

in future. Calignano *et al* (1991)<sup>16</sup> have demonstrated the reversal of the constipating effect of morphine after intraperitoneal administration of L-arginine in mice. This report and our observation suggest that L-arginine could be a useful agent for the treatment of the undesirable gastrointestinal effects associated with therapeutic administration or with the compulsive use of narcotic analgesics. Further, our observation provides a pharmacological evidence for the use of nitroglycerine in the treatment of the biliary tract effects of morphine.

It is concluded that L-arginine abolishes the biliary tract effects of morphine through the L-arginine – NO pathway and further clinical studies are needed to clarify the therapeutic use of L-arginine during the chronic use of opioids.

### Acknowledgements

The authors are thankful to Dr. F.V. Manvi, Principal, K.L.E.S's college of Pharmacy, Belgaum, India for the support and constant encouragement.

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