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European Journal of Pharmacology 460 (2003) 201–207
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# Role of opioid and nitric oxide systems in the nonadrenergic noncholinergic-mediated relaxation of corpus cavernosum in bile duct-ligated rats

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Received 28 October 2002; received in revised form 13 December 2002; accepted 19 December 2002

**Abstract**

Changes in nonadrenergic noncholinergic (NANC)-mediated relaxation of the anococcygeus muscle have been demonstrated in cholestasis. Cholestasis is also associated with accumulation of endogenous opioid peptides and nitric oxide (NO) overproduction. This study was therefore undertaken to investigate the effect of cholestasis on the NANC-mediated relaxation of corpus cavernosum in bile duct-ligated rats and to examine the possible roles of the opioid system and nitric oxide in the cholestasis-associated alterations of corpus relaxation. Bile duct-ligated and sham-operated rats were treated for 2 weeks with either normal saline, *N* (ω)-nitro L-arginine methylester (L-NAME) (3 mg/kg/day, i.p.) or naltrexone (20 mg/kg/day, i.p.). On the 14th day, the strips of corpus cavernosum were mounted under tension in a standard oxygenated organ bath with guanethidine sulfate (5 μM) and atropine sulfate (1 μM) (to produce adrenergic and cholinergic blockade). The strips were precontracted with phenylephrine hydrochloride (7.5 μM) and electrical field stimulation was applied at different frequencies to obtain NANC-mediated frequency-dependent relaxant responses. The results showed that the amplitudes of relaxation responses at each frequency in bile duct-ligated rats were greater than the responses of sham-operated animals. This increase in relaxation responses in bile duct-ligated rats was inhibited by chronic L-NAME administration for 2 weeks so it seemed that it might be due to the nitric oxide overproduction in cholestatic states. Chronic administration of naltrexone for 2 weeks to bile duct-ligated rats had the same inhibitory effect on the relaxation responses. Our results demonstrated that in cholestasis, there was an increase in NANC-mediated relaxation of corpus cavernosum and both opioid and nitric oxide systems were involved in this increase.


*Keywords:* Cholestasis; NANC (non-adrenergic non-cholinergic) relaxation; Corpus cavernosum, rat; Nitric oxide (NO); Opioid; Electrical field stimulation

## 1. Introduction

Penile erection comprises increased arterial inflow and restricted venous outflow from the penis, coordinated by relaxation of the penile corpus cavernosum (Lue and Tanagho, 1987). The relaxation of cavernosal smooth muscle is mediated by several neurotransmitters (Andersson and Wagner, 1995). Previous observations have suggested that nitric oxide (NO) or an NO-like substance appears to be the most important relaxant involved in the erection process (Andersson and Wagner, 1995; Burnett et al., 1992), which is mainly derived from nonadrenergic noncholinergic (NANC) nerves (Kim et al., 1991). We previously demonstrated that, in cholestatic rats, there was an increase in NANC-mediated relaxation of the anococcygeus muscle (Dehpour et al., 2002). Such an increase makes observable alterations in nitrergic-mediated relaxation of corpus cavernosum smooth muscle in cholestasis.

Cholestasis is associated with changes in some endogenous ligands such as opioid peptides (Swain et al., 1992) and nitric oxide (Nahavandi et al., 2001b). Our previous studies have provided evidence in favour of NO overproduction in various tissues and systems in animal models of cholestasis (Nahavandi et al., 1999, 2001a,b; Namiranian et al., 2001). Also an increased level of endogenous opioid peptides, mainly [Met$^5$]enkephalin, has been reported in cholestasis (Swain et al., 1992). In our recent study, we showed that both these systems had a role in the alterations of nitrergic neurotransmission in the anococcygeus muscles of cholestatic rats (Dehpour et al., 2002). Therefore, it is

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doi:10.1016/S0014-2999(02)02946-1

possible to observe a similar phenomenon in NANC-mediated relaxation of corpus cavernosum in cholestatic rats.

The aim of the present experiments was to investigate the effects of cholestasis on the nonadrenergic, noncholinergic (NANC)-mediated relaxation of isolated corpus cavernosum smooth muscle in bile duct-ligated rats. We have further examined the possible roles of the opioid system and nitric oxide in the cholestasis-associated alterations of corpus relaxation.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats, weighing 200–250 g, were purchased from Iran Pasteur Institute. The animals were housed in a light-controlled room with a 12-h day/night cycle and were given free access to food and water. Experiments were performed in accordance with the recommendations of the ethics committee on animal experiments of the Medical School, Tehran University of Medical Sciences.

Six experimental groups were used in this study; each group consisted of six rats: the first and the second groups were sham and bile duct-ligated groups, which were treated with normal saline (1 ml/kg/day, i.p.) for 2 weeks; the third and the fourth groups were sham and bile duct-ligated groups, which were treated with *N* (ω)-nitro L-arginine methyl ester (L-NAME) (3 mg/kg/day, i.p.) for 2 weeks; the fifth and the sixth groups were sham and bile duct-ligated groups, which were treated with naltrexone (20 mg/kg/day, i.p.) for 2 weeks.

### 2.2. Surgery

Laparotomy was performed under general anesthesia induced by intraperitoneal (i.p.) injection of ketamine HCl (50 mg/kg) and promazine HCl (10 mg/kg). In bile duct-ligated animals, the bile duct was isolated, doubly ligated and resected between the ligatures (Cameron and Oakley, 1932). In sham-operated controls, the bile duct was identified, manipulated and left in situ. Experiments were performed 14 days after surgery, when the bile duct-ligated groups had shown signs of overt cholestasis (jaundice, dark urine and steatorrhea).

### 2.3. Preparation of the rat corpus cavernosum strips

The rats were killed by cervical dislocation. Penises were removed and promptly placed in a petri dish containing Krebs-bicarbonate solution (containing in mM: NaCl: 118.1, KCl: 4.7, $KH_2PO_4$: 1.0, $MgSO_4$: 1.0, $NaHCO_3$: 25.0, $CaCl_2$: 2.5 and glucose: 11.1); bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. The glans penis and urethra were excised and the corpus cavernosum tissue was then dissected free from the tunica albuginea. Two corpus cavernosums were separated by cutting the fibrous septum between them. They were mounted separately in 20-ml organ chambers with one end tied to an electrode holder and the other to a wire connected to a force transducer (Narco F-60, Narco biosystems, Houston, TX, USA). The chambers contained Krebs-bicarbonate solution (pH 7.4) at 37 °C equilibrated with 95% oxygen and 5% carbon dioxide. The strips were allowed to equilibrate under optimal resting tension for 60 min. This optimal resting tension was calculated as follows: the strips were stretched over a range of resting tension from 2 to 20 N and after equilibration for 60 min the contractile responses to phenylephrine (7.5 μM) were measured. The optimal resting tension for corpus strips prepared in this manner was 15 N. This value was applied in all subsequent experiments from a Grass stimulator (Model S88). Electrical field stimulation was applied via two parallel platinum electrodes on either side of the corpus strips. In experiments in which electrical field stimulation was used, atropine (1 μM, to produce cholinergic blockade) and guanethidine (5 μM, to produce adrenergic blockade) were always present in the bathing medium to obtain NANC conditions.

### 2.4. Drugs

The following drugs were used: phenylephrine hydrochloride, sodium nitroprusside, *N* (ω)-nitro L-arginine methyl ester (L-NAME), guanethidine sulfate and atropine sulfate (Sigma, St. Louis, MO, USA). Naltrexone hydrochloride was a kind gift from Iran Daru, Tehran, Iran. All drugs were freshly dissolved daily in distilled water.

### 2.5. Responses to phenylephrine and sodium nitroprusside

In sham-operated and 14-day bile duct-ligated groups, concentration–response curves to phenylephrine (10 nM to 1 mM) were obtained by the cumulative addition of phenylephrine to the chamber in half-log increments. The $EC_{50S}$ of phenylephrine in two experimental groups of animals were compared.

In the next experiment, after equilibration, intact strips of corpus cavernosum from sham-operated and 14-day bile duct-ligated groups were precontracted with phenylephrine (7.5 μM; $EC_{80}$). When the contraction stabilized, concentration–response curves for sodium nitroprusside, an NO donor (1 nM to 1 mM) were obtained by the cumulative additions of sodium nitroprusside to the chamber in half-log increments and the $EC_{50S}$ of sodium nitroprusside in two experimental groups were compared.

### 2.6. Responses to electrical field stimulations

The bathing medium routinely contained, as described above, guanethidine and atropine. In sham-operated and bile duct-ligated groups treated with normal saline, strips of corpus cavernosum were precontracted with phenylephrine (7.5 μM; $EC_{80}$) and when the contraction stabilized,

electrical field stimulation (150 V, 3 ms duration at a frequency of 5 Hz, for 8 s every 120 s) induced a NANC relaxation, which was inhibited by the cumulative addition of L-NAME, an NO synthase inhibitor.

In sham-operated and bile duct-ligated groups, the frequency–response curves for electrical field stimulation were obtained, using consecutive 8 s stimulations at the frequencies of 2, 5 and 10 Hz.

### 2.7. Effect of L-NAME and naltrexone treatment

One subgroup of bile duct-ligated rats and one subgroup of sham-operated animals were treated with L-NAME (3 mg/kg, i.p.) daily for 13 days after surgery. Another subgroup of bile duct-ligated and one of sham-operated animals were treated with naltrexone (20 mg/kg, i.p.) daily for 13 days after surgery. On the 14th day, the strips of corpus cavernosum were prepared and frequency–response curves to electrical field stimulation, as described above, were obtained and the results were compared with the results from sham-operated and bile duct-ligated groups.

### 2.8. Statistical analysis

The data were expressed as means ± standard error of the mean (S.E.M). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls as post-hoc test. Statistical significance was considered when $P < 0.05$.

## 3. Results

### 3.1. Animal model of cholestasis

One day after laparotomy, bile duct-ligated rats revealed manifestations of cholestasis such as jaundice and dark urine. These manifestations were not seen in any of the sham-operated animals. Plasma levels of bilirubin were increased significantly in bile duct-ligated rats compared with sham-operated animals: 95 ± 8 and 9 ± 3 μM ($P < 0.01$), respectively. There was no significant difference in the weight increase of bile duct-ligated and sham-operated groups.

### 3.2. Responses to phenylephrine and sodium nitroprusside

There was no significant difference between the maximal contractile responses to phenylephrine in sham-operated and in bile duct-ligated groups (4173 ± 350 and 4691 ± 250 mN, respectively) or between the contractile responses to 7.5 μM phenylephrine (3528 ± 272 and 3893 ± 240 mN, respectively). In sham-operated and bile duct-ligated groups, phenylephrine caused concentration-dependent contractions in strips of corpus cavernosum (Fig. 1A). Values for the $EC_{50S}$ were not significantly different between two groups (1.13 ± 0.28 and 1.21 ± 0.23 μM, respectively).

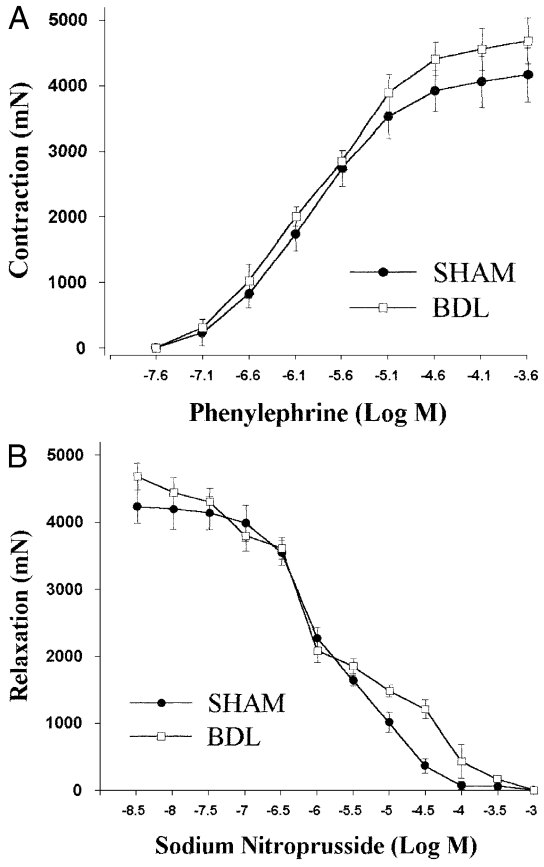


Fig. 1. (A) Concentration-dependent contraction in response to phenylephrine in isolated corpus cavernosum muscles and (B) concentration-dependent relaxation in response to sodium nitroprusside in precontracted cavernosum muscles of sham-operated (●) and bile duct-ligated (□) rats. Each group consisted of six rats.

In phenylephrine-precontracted strips of corpus cavernosum, sodium nitroprusside caused concentration-dependent relaxation (Fig. 1B). There was no significant difference between the $EC_{50S}$ for sham-operated and for bile duct-ligated groups (1.32 ± 0.26 and 0.82 ± 0.03 μM, respectively).

### 3.3. Responses to electrical field stimulation

Corpus cavernosum strips, precontracted with phenylephrine in the presence of guanethidine and atropine, were relaxed in a frequency-dependent manner by electrical field stimulation (Fig. 2). Since guanethidine and atropine blocked the adrenergic and cholinergic nerve-mediated effects of electrical stimulation, the relaxation response of cavernosum smooth muscle induced by electrical field stimulations was due to NANC mechanisms. The addition of an inhibitor of NO synthase, L-NAME, caused a concentration-dependent inhibition of the relaxation responses to 5-Hz stimulation (Fig. 3), which suggests that NO is responsible for the NANC effect in this model. The frequency-dependent nitrergic relaxant responses were significantly enhanced in the bile duct-ligated group as compared to those of

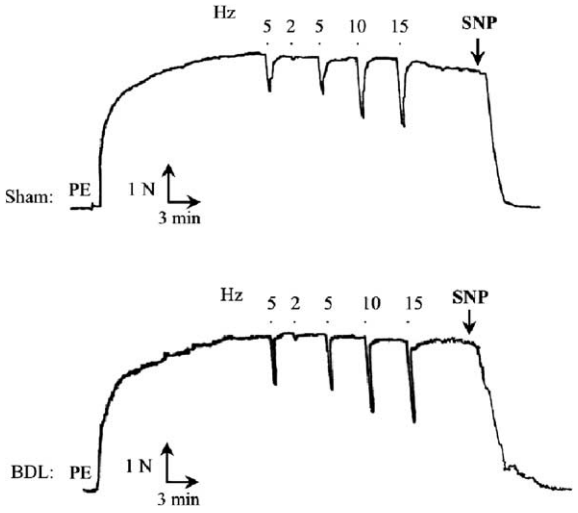


Fig. 2. Electrical field stimulation-induced frequency-dependent relaxation in cavernosal tissues precontracted with phenylephrine (7.5 μM) in the presence of guanethidine (5 μM) and atropine (1 μM). Electrical field stimulation was applied at 2, 5, 10 and 15 Hz.

sham-operated animals and interestingly, the duration of the relaxation was shorter in the bile duct-ligated group (Figs. 2 and 4).

### 3.4. Effect of L-NAME and naltrexone treatment

In the bile duct-ligated group treated chronically with L-NAME (3 mg/kg/day), the nitrergic relaxant responses to electrical field stimulation were significantly decreased when compared with those of the normal saline-treated bile duct-ligated group, but these relaxant responses were not significantly different from the relaxant responses of sham-operated animals treated chronically with either normal saline or L-NAME (Fig. 5A). In the bile duct-ligated group chronically treated with naltrexone (20 mg/kg/day), the nitrergic relaxant responses were significantly decreased

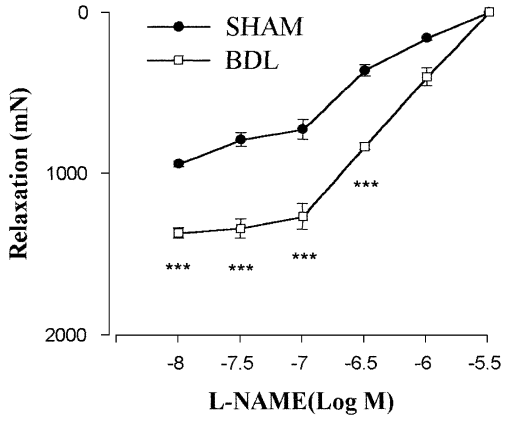


Fig. 3. L-NAME inhibited the nonadrenergic, noncholinergic relaxation of rat corpus cavernosum muscle in a concentration-dependent manner. The dose-responses are shown for sham-operated (●) and bile duct-ligated (□) rats. Each group consisted of six rats. (***$P<0.001$ compared with sham-operated group).

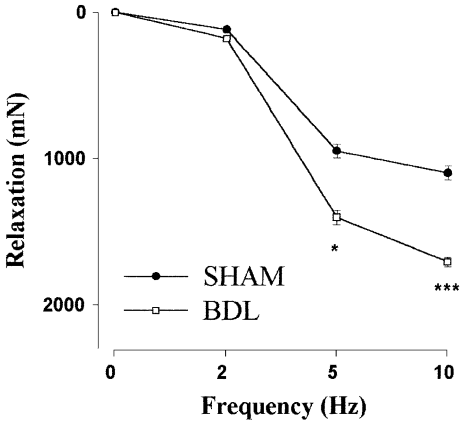


Fig. 4. Relaxation of corpus cavernosum by electrical field stimulation was significantly enhanced in bile duct-ligated groups (□) when compared with sham-operated animals (●). Each group consisted of six rats. (*$P<0.05$; ***$P<0.001$ compared with sham-operated group).

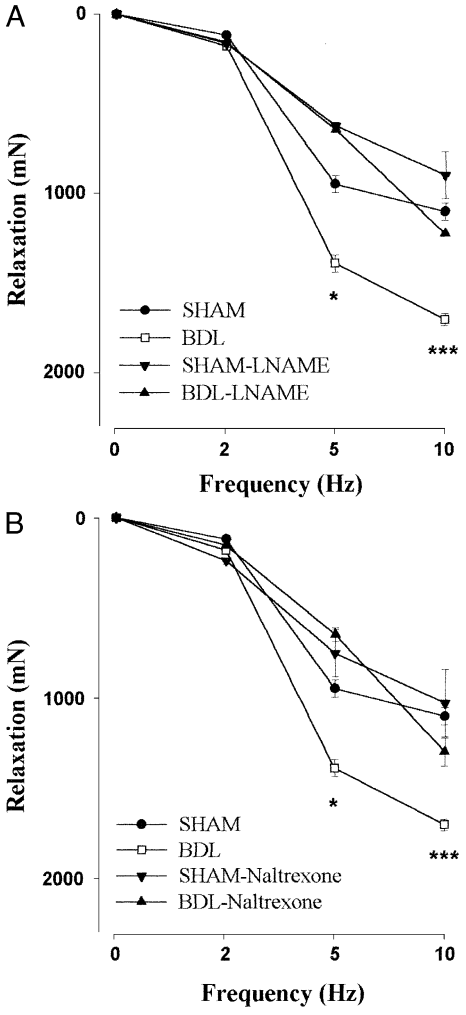


Fig. 5. The effect of chronic treatment with (A) L-NAME and (B) naltrexone on the NANC-mediated relaxation responses in sham-operated (●), bile duct-ligated (□), sham-operated animals with chronic treatment (▼) and bile duct-ligated rats with chronic treatment (▲). Each group consisted of six rats. (*$P<0.05$; ***$P<0.001$ compared with sham-operated group).

when compared with those of the normal saline-treated bile duct-ligated group, but these responses were not significantly different from the nitrergic relaxant responses of the sham-operated groups treated with either normal saline or naltrexone (Fig. 5B). In sham-operated animals treated with either L-NAME or naltrexone, the nitrergic relaxant responses were not significantly different from those of sham-operated animals treated with normal saline (Fig. 5A, B).

## 4. Discussion

In the present study, we demonstrated that the NANC-mediated relaxation of corpus cavernosum was increased in bile duct-ligated rats. Relaxation of the smooth muscle was inhibited by the cumulative addition of L-NAME, a competitive nonselective NOS inhibitor, to the medium, showing this effect to be mainly mediated via nitrergic neurotransmission. The increase in nitrergic relaxation was not due to the changes in responsiveness of the smooth muscle to nitric oxide (NO), since concentration–response curves to sodium nitroprusside, an NO donor, in sham and bile duct-ligated animals were indistinguishable. Concentration–response curves for phenylephrine were also indistinguishable between sham and bile duct-ligated animals, which excludes the possibility that the increase in nitrergic relaxation might have been due to an alteration in responsiveness of the smooth muscle to phenylephrine. The increase in nitrergic relaxation was inhibited by chronic administration of L-NAME for 2 weeks in bile duct-ligated animals. Therefore, it seems that this increase might have been due to an increased nitrergic transmission in cholestasis models.

In penile corpus cavernosum, NO is produced in the endothelium and nitrergic nerves (Moncada et al., 1997) by endothelial NO synthase (eNOS) and neural NO synthase (nNOS), respectively. However, the relaxation of the corpus cavernosum by transmural stimulation does not require a functional endothelium (Kim et al., 1991; Saenz de Tejada et al., 1988; Okamura et al., 1999). Thus, most of the NO-eliciting relaxation in penile tissue seems to be derived from neural NO synthase in nitrergic nerves (Cellek et al., 1999). Our results demonstrated an increase in NANC-mediated relaxation in cholestatic animals, so nNOS may have a role in this accentuated nitrergic transmission.

Our previous studies (Nahavandi et al., 1999, 2001a,b; Namiranian et al., 2001; Sadr et al., 1999) in accordance with others (Heinemann and Stauber, 1995; Inan et al., 1997; Niederberger et al., 1995) have suggested that there is NO overproduction in holestasis as well as in animal models of cirrhosis, but its reason is not clear. The question of which isoform of NOS is primarily responsible for the overproduction of NO in cholestasis and cirrhosis constitutes an area of major controversy. Vallance and Moncada (1991) originally proposed that iNOS upregulation secondary to endotoxaemia was the primary source of elevated NO levels following bile duct obstruction. Since then, conflicting data have emerged, suggesting that eNOS alone (Cahill et al., 1996; Wiest et al., 1999; Gadano et al., 1997; Martin et al., 1996; Hori et al., 1998), iNOS (Guarner et al., 1993), both enzymes (Morales-Ruiz et al., 1996) or neither (Fernandez et al., 1995) are upregulated in bile duct-ligated animals. Although many studies have focused on the role of eNOS and iNOS in the pathophysiology of cholestasis and cirrhosis, little if anything is known about the role of nNOS in such liver diseases. There are reports of an increased expression of genes coding for nNOS protein in chronic liver disease (Butterworth, 2000). From another study, Xu et al. (2000) reported elevated nNOS protein expression in the aorta of cirrhotic rats. They also showed that chronic treatment of cholestatic rats with a selective nNOS inhibitor, 7-nitroindazole, normalized some of the cardiovascular problems of cirrhosis. However, further studies are needed to understand the roles of nNOS in the cholestatic state and its importance in the pathophysiology of cholestasis.

As another result of this study, daily administration to bile duct-ligated rats of naltrexone, an opioid receptor antagonist, during the 2-week cholestatic period prevented the increase in nitrergic relaxation. It has been suggested that cholestatic liver disease is associated with an increased neurotransmission mediated by the opioid system and several reports have shown that, in cholestasis, there is an increase in the plasma levels of endogenous opioids, mainly [$Met^5$]enkephalin (Swain et al., 1992). So it seems that this increased opioidergic tone of cholestasis may have a role in the increased nitrergic relaxation of corpus cavernosum.

Interaction between opioids and nitric oxide has been shown in various biological models such as morphine-induced analgesia and opioid tolerance (Babey et al., 1994; Brignola et al., 1994). Opioid tolerance is generally reported to increase nNOS expression. Machelska et al. (1997) and Wong et al. (2000) separately reported that morphine tolerance increased nNOS expression in the rat spinal cord. Also, upregulation of nNOS immunoreactivity was reported in morphine-treated mice (Cuellar et al., 2000). Since cholestasis is associated with an increased opioid tone (Swain et al., 1992; Thornton and Losowsky, 1998) and state of tolerance (Ghafourifar et al., 1997; Dehpour et al., 1998, 2000), it seems possible that nNOS expression is increased in the neurons of cholestatic animals, leading to an increase in nitrergic relaxation of corpus cavernosum. Moreover, we have reported previously that, in bile duct-ligated rats, chronic treatment with naltrexone lowered the plasma levels of nitrite and nitrate (Nahavandi et al., 2001b) and this suggested that opioids might have at least a partial role in NO overproduction in cholestasis.

There is a report showing a decrease in the hypothalamic nNOS levels and activity in cholestatic rats (Swain et al., 1997), which differs from our hypothesis. That study, however, did not investigate the nNOS activity in other parts of the central nervous system (CNS) of bile

duct-ligated animals. In addition, Cuellar et al. (2000) reported that, in morphine-treated mice, there was an increase in the number of nNOS-positive cells in certain parts of the brain, such as cerebellum, locus coeruleus, olfactory bulb and medulla oblongata, but a decrease in nNOS immunoreactivity in the hypothalamus. In view of the variability in nNOS immunoreactivity of the hypothalamus in opioid models, further studies must be done to investigate the changes in nNOS activity in cholestasis.

It is of clinical importance that many studies investigated the interaction between the opioid peptides and sexual activity (Berendsen and Gower, 1986; Fabbri et al., 1989; Arletti et al., 1997; Paice et al., 1994; Clark et al., 1988; Crowley and Simpson, 1987; Cicero et al., 1975). The chronic administration of opiates has been associated with loss of libido and sexual potency in men and experimental animals (Berendsen and Gower, 1986; Fabbri et al., 1989; Arletti et al., 1997; Paice et al., 1994; Clark et al., 1988; Crowley and Simpson, 1987; Cicero et al., 1975). Fabbri et al. (1989) reported that administration of the opioid receptor antagonist, naltrexone, increased erectile activity in men with idiopathic impotence who were not given opioids, suggesting that endogenous opioid peptides have an inhibitory effect on erectile function. However, our results suggested an enhancing effect of endogenous opioid peptides on the relaxation of corpus cavernosum in bile duct-ligated rats. It is difficult to compare those studies with ours because previous studies have focused on the central effect of endogenous opioid peptides on sexual activity, while we investigated the relaxation of isolated corpus cavernosum strips. It had been demonstrated earlier that there is a difference between the central and the local effects of opioid peptides on penile erection. Clark et al. (1988) have shown that the decline in sexual behaviour induced by chronic morphine is primarily due to the failure of sexual arousal and not of erectile activity, so it seems necessary to investigate further the central effects of endogenous opioid peptides on the sexual behaviour of cholestatic subjects.

In summary, the present study demonstrated that, in cholestasis, there is an increase in nitrergic relaxation of corpus cavernosum. The present results together with our previous results with anococcygeus muscle (Dehpour et al., 2002) showed clearly that nitrergic neurotransmission is increased in both nonvascular (anococcygeus) and vascular (corpus cavernosum) smooth muscles, providing evidence of a role for both nitrergic and opioid systems in the relaxation. The activity of nNOS in tissues of cholestatic subjects and its role in the pathophysiology of cholestasis remain topics for further studies.

## Acknowledgements

The authors are grateful for the constructive comments of Dr. Khodadad Namiranian, Dr. Ali Gaskari, Dr. Houman Homayoun, Dr. Shahram Ejtemaei Mehr and Dr. Alireza Mani and also to Iran Daru for their kind gift.

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