Involvement of Nitric Oxide System on Anxiolytic-Like Behaviors Induced by Cholestasiss

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ABSTRACT

Introduction: The mechanisms of hepatic encephalopathy are not fully understood. Moreover, there is no comprehensive data concerning the effects of nitric oxide (NO) system on anxiolytic-like behaviors induced by bile duct ligation (BDL).

Methods: Male mice weighing 25-30 g were used and anxiety-like behaviors were tested using hole-board task.

Results: The data indicated that cholestasis increased the number of head-dipping but did not alter other aspects of behavior, 7 days after BDL, suggesting an anxiolytic-like response. Furthermore, the results showed that intraperitoneal (i.p.) injection of L-arginine (200 and 250 mg/kg) 15 min before testing induced anxiolytic-like behaviors in the normal animals, 4 and 7 days after BDL (considering that the dose of 200 mg in the normal mice is ineffective but is effective in the BDL mice). On the other hand, injection of L-NAME (35 and 45 mg/kg, i.p.) 15 min before testing induced anxiogenic-like behaviors in the normal animals, 4 and 7 days after BDL (the dose of 35 mg/kg in the normal mice is ineffective but is effective in the BDL mice). Moreover, injection of ineffective doses of L-NAME (25 and 35 mg/kg, i.p.) 15 min before administration of L-arginine (250 mg/kg, i.p.) and 7 days after BDL, decreased anxiolytic-like behaviors, significantly.

Discussion: Cholestatic mice show anxiolytic-like behaviors suggesting the involvement of the nitric oxide system.

1. Introduction

A reduction in canalicular bile flow causes cholestasis, which is primarily manifested as conjugated hyperbilirubinemia. The major clinical signs may be due to retention of substances which are dependent on bile flow for excretion, such as bile acids (Pak & Lee, 1993) and endotoxinemia (Inan, Sayek, Tel, & Sahin-Erdemli, 1997). Many histopathologic changes may reflect the nature and degree of the physiologic disturbance and show the pathophysiologic basis. However, the mechanisms causing hepatic encephalopathy are not completely understood. A well-known and widely used animal model for cholestasis is the bile duct ligation (BDL) which is non-reversible. Some investigations have revealed that, cholestasis decreases anxiety-like behaviors (Eslimi, Oryan, Nasehi, & Zarrindast, 2011), induces memory deficits (Cauli et al., 2009; Huang, Tiao, Tain, Chen, & Hsieh, 2009; Ma-

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gen et al., 2010; Zarrindast, Hoseindoost, & Nasehi, 2012), tremor (Chung, Wang, Tzen, & Liu, 2005) and alters the sleep pattern (Newton, 2008). It is now quite clear that, cholestasis alters the activity of all classic neurotransmitter systems such as opioidergic (Roberts, Skoulis, & James, 1987; Zhang, Zheng, Pan, & Zheng, 2004), and dopaminergic (Glaser et al., 2006; Glaser et al., 2003; Zimatkin, Baraban, & Emel'yanchik, 2008) and nitric oxide (NO) (Fernandez-Martinez, Perez-Alvarez, Tsutsumi, Shibayama, & Muriel, 2006) in mice, which may be involved in the pathophysiology of cholestasis (Bergasa, 1995)

Furthermore, NO, as a neurotransmitter in the brain (Krukoff & Khalili, 1997) is labile, with a half-life of <5 sec at body temperature. It is synthesized by a group of enzymes named NO4 synthases (NOS), which catalyze the conversion of l-arginine (L-Arg) to L-Citruline, producing NO as a by-product (Moncada, 1993). NO plays an important role in regulating many behavioral, cognitive and emotional processes such as learning, aggression, locomotion, depression and anxiety (Dzoljic, De Vries, & Dzoljic, 1997). Moreover, NO acts as a retrograde messenger, regulating neurotransmitter and neuropeptide release in an activity-dependent manner in the nervous system (Hanbauer, Wink, Osawa, Edelman, & Gally, 1992). It is a signaling molecule in the brain and has been implicated in neurotransmission, synaptic plasticity, learning, perception of pain, aggression (Esplugues, 2002), anxiety and depression (Almeida, Felisbino, Lopez, Rodrigues, & Gabilan, 2006). Activation of NO-producing neurons has been shown after aversive stimuli, such as restraint stress; exposure to the elevated plus maze; and exposure to a live cat (Beijamini & Guimaraes, 2006b). The actual role of NO in anxiety is still unclear. Earlier studies indicated that NO may play an important role in mediating the anxiolytic effects of benzodiazepines, implicating a functional role of NO in relief of anxiety (Li & Quock, 2001). Furthermore, the elevated plus maze showed that systemic administration of NOS inhibitors are capable of suppressing anxiety in the EPM (Guimaraes, Beijamini, Moreira, Aguiar, & de Lucca, 2005).

Thus, according to previous studies, the aim of the present study was to evaluate the effect of L-arginine (precursor of NO) and N-nitro-l-arginine methyl ester (L-NAME; non-selective NOS inhibitor) on the anxiolytic-like behaviors induced by cholestasis in the model BDL-mice.

2. Methods

2.1. Animals

Male albino NMRI mice (Pasteur Institute; Tehran, Iran) weighing 25-30 g at time of surgery were used. Animals were kept in an animal house with a 12/12-h light-dark cycle and controlled temperature (22±2ºC). Animals were housed in groups of 10 in Plexiglas cages and food and water were available ad libitum. Ten animals were used in each group; each animal was used only once . Behavioral experiments were done during the light phase of the light/dark cycle. Eight animals were used in each experiment. All procedures were carried out in accordance with institutional guidelines for animal care and use.

2.2. Surgical Procedure

There were three experimental groups: unoperated, sham operated and BDL-mice. Laparotomy was performed under general anesthesia induced by an intra-peritoneal injection of ketamine hydrochloride (50 mg/kg) plus xylazine (5 mg/kg). Sham operation consisted of laparotomy and bile duct identification and manipulation without ligation or resection. In the BDL group the main bile duct was first ligated using two ligatures approximately 0.5 cm apart and then transected at the midpoint between the two ligatures (Bergasa et al., 1994). In the immediate postoperative period each animal was placed in a cage by itself to prevent wound dehiscence and was moved to its original cage 4 h after surgery. Operative mortality was less than 6%.

2.3. Hole-Board Apparatus

The hole-board test as a simple method for examining the response of an animal to an unfamiliar environment was first introduced by Boissier and Simon (Boissier & Simon, 1962). This test has been used to evaluate emotionality, anxiety and/or responses to stress in animals (Rodriguez Echandia, Broitman, & Foscolo, 1987). Different behaviors which can be observed and measured in this test, makes the comprehensive description of the animal's behavior possible.

The hole-board apparatus (Borj Sanat Co, Tehran, Iran) consisted of gray Perspex panels (40 cm×40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor, was made on the basis of methods used previously (Vinade et al., 2003). The board

was positioned 15 cm above a table. The animals were placed singly in the center of the board facing away from the observer and head-dip numbers were recorded by photocells arranged below the holes over 5 min. Furthermore, locomotor activity was measured by an observer unaware of the treatments. For this purpose, the ground area hole-board was divided into four equal sized squares. Locomotion was measured as the number of locomotor activity crossings from one square to another. Other behavioral performances such as latency to the first head-dipping, rearing, grooming and defecation were recorded manually by an experimenter during the test.

2.4. Drugs

The drugs used in the present study were ketamine and xylazine (Alfasan Chemical Co, Woerden, Holland), L-arginine and L-NAME (Tocris Cookson, Bristol, UK) which were dissolved in sterile saline 0.9% just before the experiment. Control animals received saline.

2.5. Experimental Design

Ten animals were used in each experimental group. The experiments were based on previous studies in order to obtain a maximum response (Zarrindast, Asgari-Afshar, & Sahebgharani, 2007; Zarrindast, Askari, Khalilzadeh, & Nouraei, 2006). The protocol has been summarized in table 1.

Table 1. Summary of experimental design

Figure	Firstly injection (i.p.)			Secondly injection (i.p.)	Effect upon specific behavior		
	Saline (ml/kg)	NAME (mg/kg)	L-arginine (mg/kg)	L-arginine (mg/kg)	Head dips (panel A)	Latency to Head dips (panel B)	Locomotor activity (panel C)
1	Time course response of cholestasis				Increase	NO effect	NO effect
Left (non cholestasis)	10	-	150-250	-	Increase	Decrease	NO effect
Medium (4 day after BDL)	10	-	150-250	-	Increase	Decrease	Decrease
Right (7 day after BDL)	10	-	150-250	-	Increase	NO effect	NO effect
Left (non cholestasis)	10	25-45	-	-	Decrease	NO effect	NO effect
Medium (4 day after BDL)	10	25-45	-	-	Decrease	NO effect	NO effect
Right (7 day after BDL)	10	25-45	-	-	Decrease	NO effect	NO effect
(7 day after BDL)	10	25-35	-	250	Decrease	NO effect	NO effect

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2.5.1. Experiment 1: effect of cholestasis on exploratory behaviors (time course response of cholestasis).

Twelve groups of mice have been used in this experiment. Four, seven, ten and thirteen days after BDL, the exploratory behaviors of animals were tested. The data from cholestatic animals were compared to the respective non-operated (normal) and operated (sham) animals groups.

2.5.2. Experiment 2: effects of L-arginine on exploratory behaviors in the presence or absence of cholestasis.

In this experiment, twelve groups of animals were used. Four groups received saline (10 ml/kg, i.p.) or different doses (150, 200 and 250 mg/kg, i.p.) of L-arginine 15 minute before the test. Two other four groups received saline (10 ml/kg, i.p.) or different doses (150, 200 and 250 mg/kg, i.p.) of L-arginine 15 minute before testing, 4 and 7 days after BDL.

2.5.3. Experiment 3: effects of L-NAME on exploratory behaviors in the presence or absence of cholestasis.

In this experiment, twelve groups of animals were used. Four groups received saline (10 ml/kg, i.p.) or different doses (25, 35 and 45 mg/kg, i.p.) of L-NAME 15 minutes before the test. Two other four groups received saline (10 ml/kg, i.p.) or different doses (25, 35 and 45 mg/kg, i.p.) of L-NAME 15 minutes before testing, 4 and 7 days after BDL.

2.5.4. Experiment 4: effects of L-NAME plus L-arginine on exploratory behaviors in the presence or absence of cholestasis.

In this experiment, ten groups of animals were used. Three groups (operated, non-operated and 7 days after BDL) received saline (10 ml/kg, i.p.) 15 minutes before testing. The other three groups received L-arginine (250 mg/kg, i.p.) or L-NAME (25, 35 mg/kg, i.p.) 15 minutes before testing. The last four groups received L-NAME (25, 35 mg/kg, i.p.) 15 minutes before injection of L-arginine (250 ml/kg, i.p.), in the seven days cholestatic animals and non-cholestatic groups.

2.7. Statistical Analysis

Since data displayed normality of distribution and homogeneity of variance, the results were statistically evaluated by analysis of variance one-way (ANOVA), in which mean ± SEM of experimental groups on the test day were compared. Further analyses for individual between-groups comparisons were carried out with post hoc Tukey's test. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Induction of Cholestasis

One day after BDL, the animals showed signs of cholestasis (jaundice, dark urine and steatorrhea).

3.2. Time Course Response of Cholestasis on Exploratory Behaviors

Figure.1. indicates the time effect of cholestasis on exploratory behaviors. One-way ANOVA and post hoc Tukey's analysis revealed that number of head dips have been increased in the 7th day ($P < 0.01$), but did not alter in the 4, 10 and, 13 days ($P > 0.05$) after BDL [$F (11, 84) = 9.97$, $p < 0.001$], respectively (Fig.1A). Moreover, there was a significant decrease in the number of head dips ($P < 0.05$) on the fourth the day after the surgery in operated groups (sham) compared to the unoperated control, demonstrating thean effect of surgery and anesthetic drugs on the anxiety-like behavior. Furthermore, One-way ANOVA and post hoc Tukey's analysis indicated that other aspects of behaviors such as latency to head dipping [$F (11, 84) = 0.71$, $P > 0.05$] (Fig, 1B), locomotor activity [$F (11, 84) = 2.95$, $P > 0.05$] (Fig, 1C), number of rearing [$F (11, 84) = 0.26$, $P > 0.05$],

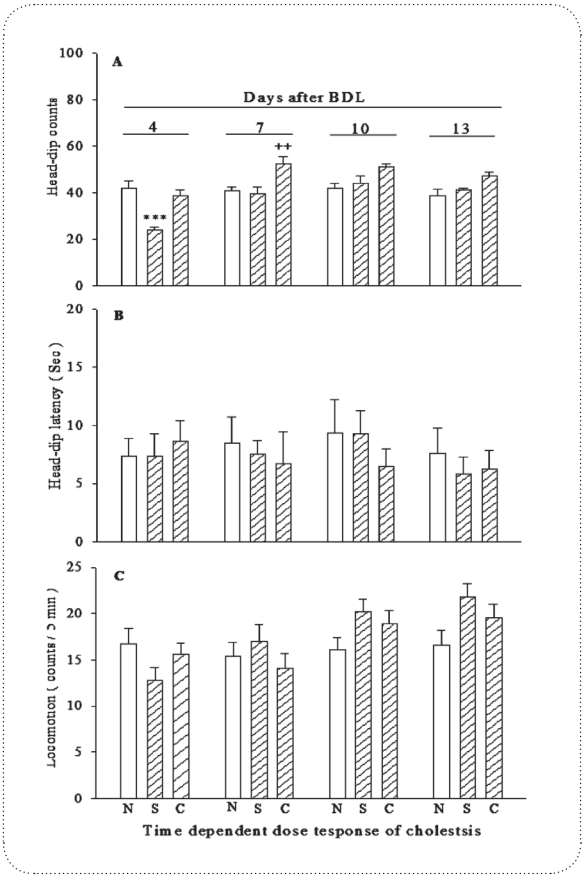

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Figure 1. The time course response of cholestasis on exploratory behaviors. Twelve groups of animals have been used for this experiment. The exploratory behaviors-induced by cholestasis have been tested 4, 7, 10 and 13 day after BDL. The exploratory behaviors including number of head dips (panel A), latency to head dipping (panel B) and locomotor activity (panel C) have been shown. Each bar is mean±S.E.M. ***P<0.001 when compared to non-operated/4 day after BDL group and ++P <0.01 when compared to non-operated /7 day after BDL group.

number of grooming [$F (11, 84) = 1.16$, $P > 0.05$] and number of defecation [$F (11, 84) = 2.1$, $P > 0.05$] were not altered. Data for rearing, grooming and defecation are not shown. In conclusion, BDL-animals showed the anxiolytic-like behaviors, 7 days after cholestasis.

3.3. Effects of L-arginine on exploratory behaviors in presence or absence of cholestasis.

One-way ANOVA and post hoc Tukey's analysis revealed that administration of different doses of L-arginine (150, 200 and 250 mg/kg, i.p.), 15 min before testing, increased the number of head-dips [F (3, 28) = 41.80, P < 0.001] (Fig.2A; left panel), but had no effect on other exploratory behaviors such as latency to head-dipping [F (3, 28) = 0.05, P > 0.05] (Fig.2B; left panel), locomotor activity [F (3, 28) = 0.2, P > 0.05] (Fig.2C; left panel), number of rearing [F (3, 28) = 0.5, P > 0.5], number of grooming [F (3, 28) = 0.27, P > 0.5] and number of defecation [F (3, 28) = 1.7, P > 0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, the data showed that L-arginine induce anxiolytic-like behaviors.

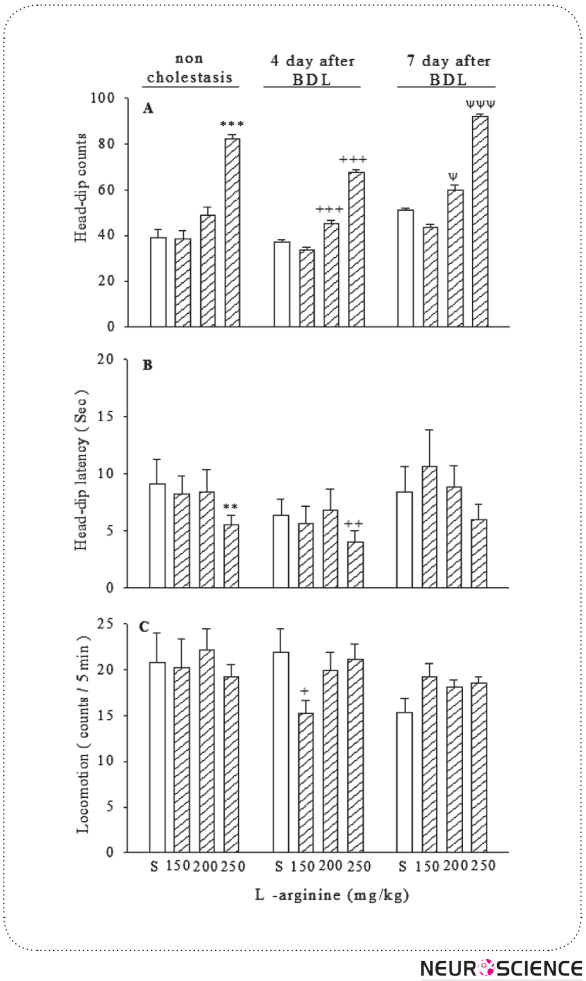

Figure 2. The effects of L-arginine on exploratory behaviors in the presence and absence of cholestasis. The figure shows the effects of pre-test administration of L-arginine (150, 200 and 250 mg/kg, i.p.) on animals which were non-cholestatic (left panel), 4 day after BDL (middle panel) or 7 day after BDL (right panel).The exploratory behaviors including number of head dips (panel A, left panel; dose response of L-arginine., panel A, middle panel; effects of L-arginine 4 day after BDL and panel A, right panel; effects of L-arginine 7 day after BDL), latency to head dipping (panel B, left panel; dose response of L-arginine., panel B, middle panel; effects of L-arginine 4 day after BDL and panel B, right panel; effects of L-arginine 7 day after BDL) and locomotor activity (panel C, left panel; dose response of L-arginine., panel C, middle panel; effects of L-arginine 4 day after BDL and panel C, right panel; effects of L-arginine 7 day after BDL) have been showed. Each bar is mean±S.E.M. ***P<0.001 when compared to saline/non-cholestatic mice groups, +++P <0.001 when compared to saline/4 day after BDL group and P <0.05, P <0.001when compared to saline/7 day after BDL group.

Furthermore, the results indicated that administration of L-arginine (150, 200 and 250 mg/kg, i.p.), 15 min before testing, 4 days after BDL increases the number of head-dips [F (3, 28) = 157.85, P < 0.001] (Fig.2A; middle panel) and, decreasesd latency to head-dipping [F (3, 28) = 2.9, P < 0.05] (Fig.2B; middle panel) but hasd no effect on other exploratory behaviors such as locomotor activity [F (3, 28) = 1, P > 0.05] (Fig.2C; middle panel), number of rearing [F (3, 28) = 1.9, P > 0.5], number of grooming [F (3, 28) = 0.15, P > 0.5] and number of defecation [F (3, 28) = 1.9, P >0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, the data showed that L-arginine (250 mg/kg, i.p.) increased anxiolytic-like behaviors 4 days after BDL.

Moreover, the results indicated that administration of L-arginine (150, 200 and 250 mg/kg, i.p.), 15 min before testing, 7 days after BDL increased number of head-dips [F (3, 28) = 94.6, P < 0.001] (Fig.2A; right panel) but had no effect on other exploratory behaviors such as latency to head-dipping [F (3, 28) = 0.7, P > 0.05] (Fig.2B; right panel), locomotor activity [F (3, 28) = 2.5, P > 0.05] (Fig.2C; right panel), number of rearing [F (3, 28) = 2.1, P > 0.5], number of grooming [F (3,28) = 0.4, P > 0.5] and number of defecation [F (3, 28) = 0.7, P >0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, two doses of L-arginine (200 (subthreshold) and 250 (effective) mg/kg, i.p.) increased anxiolytic-like behaviors 4 and 7 days after BDL

3.3. Effects of L-NAME on exploratory behaviors in the presence or absence of cholestasis.

One-way ANOVA and post hoc Tukey's analysis revealed that administration of different doses of L-NAME (25, 35 and 45 mg/kg, i.p.), 15 min before testing decreased the number of head-dips [F (3, 28) = 5.34, P < 0.01] (Fig.2A; left panel) but had no effect on other exploratory behaviors such as latency to head-dipping [F (3, 28) = 0.35, P > 0.05] (Fig.2B; left panel),

locomotor activity [F (3, 28) = 0.6, P > 0.05] (Fig.2C; left panel), number of rearing [F (3, 28) = 0.06, P > 0.5], number of grooming [F (3, 28) = 0.5, P > 0.5] and number of defecation [F (3, 28) = 2.2, P >0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, the data showss L-NAME induced anxiogenic-like behaviors.

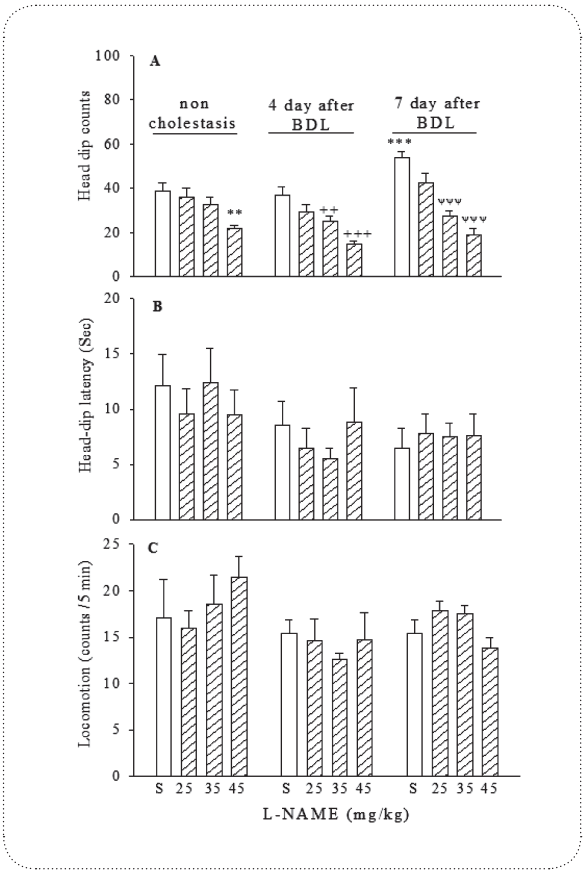

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Figure 3. The effects of L-NAME on exploratory behaviors in the presence and absence of cholestasis. The figure shows the effects of pre-test administration of L-NAME (25, 35 and 45 mg/kg, i.p.) on animals which were non-cholestatic (left panel), 4 day after BDL (middle panel) or 7 day after BDL (right panel).The exploratory behaviors including number of head dips (panel A, left panel; dose response of L-NAME., panel A, middle panel; effects of L-NAME 4 day after BDL and panel A, right panel; effects of L-NAME 7 day after BDL), latency to head dipping (panel B, left panel; dose response of L-NAME., panel B, middle panel; effects of L-NAME 4 day after BDL and panel B, right panel; effects of L-NAME 7 day after BDL) and locomotor activity (panel C, left panel; dose response of L-NAME., panel C, middle panel; effects of L-NAME 4 day after BDL and panel C, right panel; effects of L-NAME 7 day after BDL) have been showed. Each bar is mean±S.E.M. **P<0.01 when compared to saline/non-cholestatic mice group, ++P <0.01 and +++P <0.001 when compared to saline/4 day after BDL group and P <0.001when compared to saline/7 day after BDL group.

Furthermore, the results indicated that administration of L-NAME (25, 35 and 45 mg/kg, i.p.), 15 min before testing,, 4 days after BDL decreased number of head-dips [F (3, 28) = 10.88, P < 0.001] (Fig.2A; middle panel) but had no effect on other exploratory behaviors such as latency to head-dipping [F (3, 28) = 1.15, P > 0.05] (Fig.2B; middle panel), locomotor activity [F (3, 28) = 1.56, P > 0.05] (Fig.2C; middle panel), number of rearing [F (3, 28) = 0.17, P > 0.5], number of grooming [F (3, 28) = 1.24, P > 0.5] and number of defecation [F (3, 28) = 1.03, P >0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, the data showss L-NAME (35 and 45 mg/kg, i.p.) decreased anxiolytic-like behaviors 4 days after BDL and showed that anxiogenic-like behaviors.

Moreover, the results indicated that the administration of L-NAME (25, 35 and 45 mg/kg, i.p.), 15 min before testing, 7 days after BDL decreased the number of head-dips [F (3, 28) = 22.57, P < 0.001] (Fig.2A; right panel), but had no effect on other exploratory behaviors such as latency to head-dipping [F (3, 28) = 0.1, P > 0.05] (Fig.2B; right panel), locomotor activity [F (3, 28) = 0.23, P> 0.05] (Fig.2C; right panel), number of rearing [F (3, 28) = 0.5, P > 0.5], number of grooming [F (3, 28) = 0.9, P > 0.5] and number of defecation [F (3, 28) = 1.33, P >0.5]. Data for rearing, grooming and defecation are not shown. In conclusion, the two doses of L-NAME (35 (subthreshold) and 45 (effective) mg/kg, i.p.) decreased anxiolytic-like behaviors 7 days after BDL and indicated anxiogenic-like behaviors.

3.4. Effects of L-NAME plus L-arginine on exploratory behaviors in the presence or absence of cholestasis

Fig.4. One-way ANOVA and post hoc Tukey's analysis revealed that administration of L-NAME plus L-arginine, with an 15 minutes interval, 15 minutes before testing decreased the number of head dips, seven days after BDL [F (9, 70) = 58.30, p< 0.001] (Fig, 4A) compared to the respective group. On the other hand, One-way ANOVA and post hoc Tukey's analysis indicated that other aspects of behaviors such as latency to head dipping [F (9, 70) = 1.12, p> 0.05] (Fig, 4B), locomotors activity [F (9, 70) = 0.22, p> 0.05] (Fig, 4C), number of rearing [F (9, 70) = 0.84, p> 0.05], number of grooming [F (9, 70) = 01.46, p> 0.05] and number of defecation [F (9, 70) = 0.73, p> 0.05] were not altered. In conclusion, two doses of L-NAME (35(subthreshold) and 45 (effective) mg/kg, i.p.) plus L-arginine (250 mg/kg, i.p.) 7 days after BDL decreased anxiolytic-like behaviors induced by l-arginine in cholestatic mice suggesting that

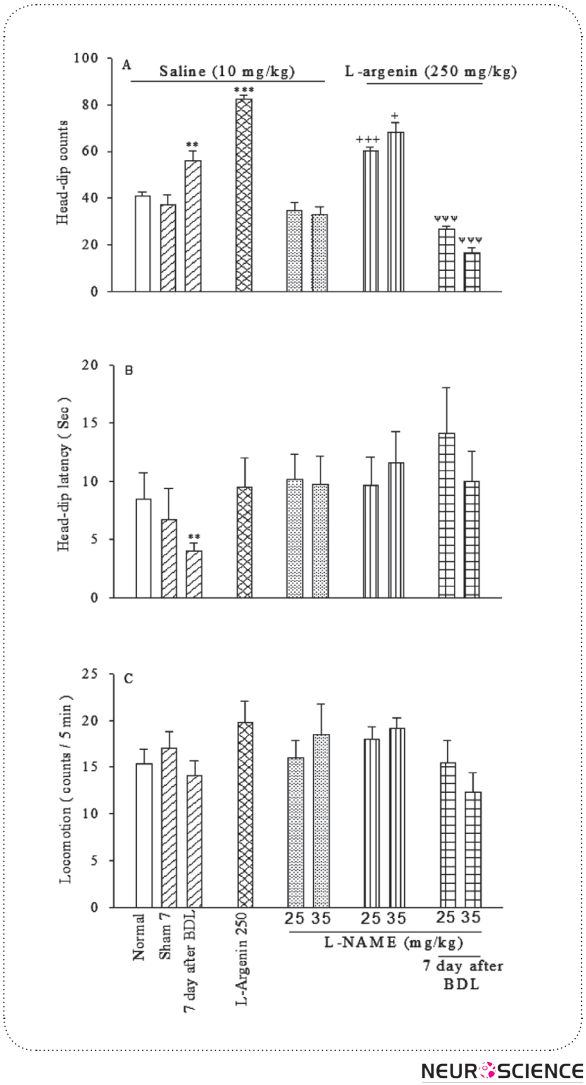

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Figure 4. The effects of L-NAME on exploratory behaviors induced by L-arginine 7 day after BDL. In this experiment, ten groups of animals were used. The exploratory behaviors of three groups of animals, non operated, operated and 7 day after BDL have been tested. Three groups of animals received L-arginine (250 mg/kg, i.p.) and two doses of L-NAME (25 and 35 mg/kg, i.p.) 15 minute before the test. Another two groups received two doses of L-NAME (25 and 35 mg/kg, i.p.) 15 minute before injection of L-arginine (250 mg/kg, i.p.). The last two groups received two doses of L-NAME (25 and 35 mg/kg, i.p.) 15 minute before injection of L-arginine (250 mg/kg, i.p.) 7 day after BDL. The exploratory behaviors including number of head dips (panel A), latency to head dipping (panel B) and locomotor activity (panel C) have been showed. Each bar is mean±S.E.M. **P<0.01 and ***P<0.001 when compared to non-operated/saline mice group, +P <0.05 and +++P <0.001 when compared to L-arginine/saline group and P<0.001when compared to L-NAME/L-arginine group.

cholestasis- induced anxiolytic-like behaviors may act through increase of NO level. Data for rearing, grooming and defecation are not shown.

4. Discussion

The present study was carried out to determine the involvement of nitric oxide (NO) in the development of anxiolytic-like behaviors induced by cholestasis. Based on previous studies, indicating an increase in the NO levels in the cholestatic animals and modulatory roles of NO in the anxiety-like behaviors, we hypothesized that cholestatic animals may show anxiolytic-like behaviors. Therefore, we examined the contribution of NO in this hypothesis.

The present data indicate that the bile duct-ligated mice (BDL-mice) on the seventh day but not four, ten and thirteen days after bile duct-ligation induced anxiolytic-like behaviors. However, the data about this cholestasis induced phenomenon are very little, our previously published data, the present results demonstrated that, cholestasis in rats (13 but not 10 days post BDL) induces anxiolytic-like behaviors while does not alter locomotor activity in the elevated plus maze test (Eslimi et al., 2011).

It has been proposed that even short-term biliary obstruction is associated with significant alterations in neurotransmission in the brain (Burak, Le, & Swain, 2001; Rioux, Le, & Swain, 2001). Sickness behaviors such as fatigue, lethargy, anorexia, fever, hypersomnia and loss of social interest can be induced following the central neurotransmission alterations which are associated with the systemic inflammatory response (reviewed in (Kent, Bluthe, Kelley, & Dantzer, 1992)). These manifestations are commonly observed in patients with biliary obstruction, and also in experimental animal models of obstructive cholestasis (McCullough, Takahashi, Le, Pittman, & Swain, 2000; Rioux et al., 2001; Swain & Maric, 1997).

Furthermore, it has been shown that the endogenous opioid system undergoes significant changes during cholestasis (Swain et al., 1992). The mechanical obstruction in the BDL-mice has been used to induce a cholestatic state in rodents. This model makes us able to investigate the changes in opioid activity, along with the involvement of NO pathway. The mechanism of the increased level of endogenous opioids (Jones & Bergasa, 2000; Jones, Neuberger, & Bergasa, 2002) and NO (Fernandez-Martinez et al., 2006) following cholestasis is not yet completely understood. There is a suggestion

that both overproduction of endogenous opioids and protection of these peptides from degradation may be involved in the elevation of total opioid activity (Swain et al., 1992).

The results obtained in the present investigation indicate that pre-test administration of different doses of L-arginine (a NO precursor) in the non-cholestatic mice increased number of head-dips, but did not alter latency to head dip and locomotor activity, suggesting anxiolytic-like behaviors. On the other hand, pre-test administration of different doses of L-NAME (a non-selective NOS-inhibitor) in the non-cholestatic mice decreased number of head-dips but not the latency to head dip and locomotor activity, showing anxiogenic-like behaviors. These findings are in line with previous reports on the involvement of NO system in the modulation of anxiety (Beijamini & Guimaraes, 2006a; Faria et al., 1997; Guimaraes et al., 2005; Moncada, Palmer, & Higgs, 1991; Volke et al., 1995). Nitric oxide, as a neuronal messenger and a modulator of neurotransmitters in the central nervous system (Moncada et al., 1991), has been implicated in the regulation of anxiety (Czech, Jacobson, LeSueur-Reed, & Kazel, 2003; Faria et al., 1997; Vale, Green, Montgomery, & Shafi, 1998). These results are in agreement with previous studies that systemic (Czech et al., 2003; De Oliveira, Del Bel, & Guimaraes, 1997; Monzon, Varas, & De Barioglio, 2001; Pokk & Vali, 2002; Quock & Nguyen, 1992; Vale et al., 1998) and intra-hippocampal (Monzon et al., 2001; Roohbakhsh, Moghaddam, Massoudi, & Zarrindast, 2007) injections of NOS-inhibitors induced anxiogenic effect in the plus-maze test, or antagonized the anxiolytic effect of nitrous oxide (Li & Quock, 2001) and chlordiazepoxide (Li & Quock, 2001; Quock & Nguyen, 1992) or reduced open-arm activity in the elevated plus-maze (De Oliveira et al., 1997; Monzon et al., 2001; Vale et al., 1998). However, there are other studies suggesting that NOS inhibitors decreased anxiety (Dunn, Reed, Copeland, & Frye, 1998; Eroglu & Caglayan, 1997; Faria et al., 1997; Forestiero, Manfrim, Guimaraes, & de Oliveira, 2006; Guimaraes et al., 2005; Volke et al., 1995; Volke et al., 1997; Wiley, Cristello, & Balster, 1995; Yildiz, Ulak, Erden, & Gacar, 2000). Furthermore, Shin et al. have shown that L-arginine inhibits and L-NAME enhances the anxiolytic effect of acute morphine in mice (Shin, Kim, Swanson, Hong, & Oh, 2003). There are also reports indicating that neither systemic nor i.c.v. administration of l-arginine affect anxiety-behaviors in the EPM (Faria et al., 1997; Volke et al., 1997). The reasons for these contradictory results are not clear, but may be due to different experimental procedures and routes of administration (Monzon et al., 2001). Nitric oxide may modulate motor function in the central nervous system (for review see reference (Del Bel et al., 2005)). Locomotor abilities have been impaired in the mice mutant for the neuronal NOS isoform (Kriegsfeld et al., 1999). The rodents treated with various NOS inhibitors also show impaired fine motor control and catalepsy (Araki et al., 2001). Therefore, alteration in the NO levels may secondarily interfere with animal models that are sensitive to anxiolytic drugs (De Oliveira et al., 1997) . The contradictory results induced by NO agents in anxiety may be due to complex interactions of NO with several neurotransmitter systems. Although NO can increase the release of glutamate, which in turn could elicit a facilitatory role in defensive reactions (Moreira, Molchanov, & Guimaraes, 2004),; its excessive production may negatively modulate NMDA function. However, an increase in cGMP or administration of NO donor may decrease the glutamate release. Furthermore, a biphasic effects for NO on gamma-aminobutyric acid and serotonin release depending on local NO and antioxidant concentrations have been indicated (Trabace & Kendrick, 2000).

The present results indicate that pre-test administration of different doses of L-arginine in the BDL-mice (4 and 7 days after BDL) increased the number of head-dips but did not affect head dip latency and locomotor activity induced by cholestasis, suggesting anxiolytic-like behaviors. In addition, an ineffective dose of L-arginine (200 mg/kg) in the non-BDL mice, showed anxiolytic-like behaviors in the BDL-mice. Furthermore, pre-test administration of different doses of L-NAME in the BDL-mice (4 and 7 day after BDL) decreased the number of head-dips but did not change head dip latency and locomotor activity induced by cholestasis, showing anxiogenic-like behaviors. Thus, it should also be noted that the ineffective dose of L-NAME (35 mg/kg) in non-BDL mice revealed anxiogenic-like behavior in the BDL mice. Moreover, pre-test administration of different ineffective doses of L-NAME, 15 minute before injection of effective dose of L-arginine (250 mg/kg), 7 day after BDL decreased the number of head-dips but not head dip latency and locomotor activity. These data may further support the involvement of NO in the increased response induced in the BDL-mice. The results can be in agreement with other investigators indicating that the level of serum nitric oxide (NO) has been increased in the cholestasis conditions (Mayoral et al., 1999; Rodrigo, Alonso, Fernandez, Serrano, & Lopez, 2000). It has been suggested that histological liver injuries reduce portal flow, increases plasma NO, and liver lipid peroxidation associated with extrahepatic cholestasis. Vallance and Moncada (Vallance & Moncada, 1991) proposed the theory of NO overproduction in cir-

rhosis, while some other studies have not supported it (Fernandez et al., 1995; Sogni et al., 1992).

NO can be made from L-arginine by a family of enzymes called NO synthases (NOS), which exists in three isoforms. One isoform of NOS is involved in immunological reactions and is, activated by factors released in pathological events, such as cytokines that can induce NO and is named inducible NOS (iNOS). Two other forms of NOS are present in endothelium (endothelial NOS-eNOS) and in neurons (neuronal NOS-nNOS) (Guix, Uribesalgo, Coma, & Munoz, 2005; Lamas, Marsden, Li, Tempst, & Michel, 1992; Mungrue, Bredt, Stewart, & Husain, 2003; Prast & Philippu, 2001). Controversial suggestions exist about the involvement of different kinds of NOS in the overproduction of NO in cholestasis and cirrhosis. Vallance and Moncada (Vallance & Moncada, 1991) indicated that the primary sources of elevated NO levels following bile duct obstruction were due to iNOS up-regulation secondary to endotoxaemia. Other reports propose that eNOS alone(Cahill, Redmond, Hodges, Zhang, & Sitzmann, 1996; Gadano et al., 1997; Hori, Wiest, & Groszmann, 1998; Martin et al., 1996; Wiest, Shah, Sessa, & Groszmann, 1999), iNOS (Guarner et al., 1993), both enzymes or neither (Fernandez et al., 1995) are upregulated in BDL animals. In spite of different studies that have focused on the role of eNOS and iNOS in the pathophysiology of cholestasis and cirrhosis, little is known about the role of nNOS in such liver diseases.

However, there are reports indicating an increased expression of genes coding for nNOS protein in chronic liver disease (Butterworth, 2000; Xu et al., 2000) as well as elevated nNOS protein expression in the aorta of cirrhotic rats. They indicated that chronic treatment of cholestatic rats with a selective nNOS inhibitor, 7-nitroindazole, normalized some of the cardiovascular problems of cirrhosis. Other investigation showed that treatment with aminoguanidine, a selective inducible nitric oxide synthase (iNOS) inhibitor, restored the changes seen in cholestasis. This finding is in favor of the hypothesis that the dysfunction seen in cholestasis may be due to the negative feedback of NO on NOS activity.

In conclusion, the present results may indicate that cholestasis- induced anxiolytic-like behaviors may act through increase of NO level.

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