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# Possible involvement of cholinergic and opioid receptor mechanisms in fluoxetine mediated antinociception response in streptozotocin-induced diabetic mice

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**Abstract**

Clinical and experimental studies have been reported that antidepressant drugs can be used as co-analgesics in the management of neuropathic pain. However, the mechanism through which they alleviate pain still remains unclear. The aim of the present study was to investigate the possible mechanism of action of fluoxetine-induced antinociceptive effect in streptozotocin-induced diabetic mice, especially the involvement of non-serotonergic neurotransmitters and their receptors. Diabetes was induced in male Laka mice with a single intraperitoneal injection of streptozotocin (200 mg/kg). Four weeks after streptozotocin, diabetic mice were tested for pain responses in the tail-immersion and hot-plate assays. Diabetic mice exhibited significant hyperalgesia as compared with control mice. Fluoxetine (10 and 20 mg/kg, i.p) injected into diabetic mice produced an antinociceptive effect in both tail-immersion and hot-plate assays. The antinociceptive effect of fluoxetine in diabetic mice was significantly lower as compared with that in control mice. Pretreatment with a muscarinic receptor antagonist, atropine (2 and 5 mg/kg, i.p) and an opioid receptor antagonist, naloxone (2 and 5 mg/kg, i.p), but not the $\alpha_2$-adrenoreceptor antagonist, yohimbine (2 and 5 mg/kg, i.p) reversed the antinociceptive effect of fluoxetine (20 mg/kg). These results suggest that apart from serotonin pathway, muscarinic and opioid receptors also participate in fluoxetine-induced antinociception in diabetic neuropathic pain.


*Keywords:* Atropine; Hot-plate; Fluoxetine; Naloxone; Neuropathic pain; Streptozotocin; Tail-immersion

## 1. Introduction

Neuropathic pain is generally considered to be one of the most common complications of diabetes, affecting both types of diabetes equally (Watkins, 1990; Vinik et al., 1992; Clark and Lee, 1995). It is mostly characterized by pain which can occur spontaneously as a result of exposure to normally mildly painful stimuli, i.e. hyperalgesia (Brown and Asbury, 1984). Although hyperglycaemia (Greene et al., 1987), neuronal loss (Dyck et al., 1985; Said et al., 1992) or neurotransmitter changes (Bitar et al., 1985; Chu et al., 1986; Bellush and Reid, 1991) have been reported to be responsible for the change in pain perception, the exact aetiological factors involved are still under investigation.

The behavioural reaction to hyperalgesia has been described in animal models of diabetes (Kamei et al., 1991; Courteix et al., 1993; Anjaneyulu and Chopra, 2003). Streptozotocin-induced diabetic mice have been widely used as a model of diabetes mellitus, and a number of anomalies in pain perception have been demonstrated in this model (Kamei et al., 2000). Chemical-induced flinching, thermal hyperalgesia and allodynia have been observed in streptozotocin-treated mice (Ohsawa and Kamei, 1999; Kamei et al., 2001).

The neurotransmitter 5-hydroxytryptamine (5-HT) is widely accepted as an important participant in the brain and spinal inhibition of nociceptive transmission (Bardin et al., 2000; Zhang and Wu, 2000). Behavioural studies have demonstrated that 5-HT is implicated in the control exerted by the brain on nociception either by afferent fibre hyperpolarization or through a presynaptic action. It has been reported that destruction of serotonergic projection is known to greatly affect nociception. Serotonergic deficiency is a common factor in both mental depression and chronic pain (Vogel et al., 2003). It has been reported that destruction of serotonergic projections greatly

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affects nociception. In contrast, increasing the availability of 5-HT at the synapse is reported to inhibit nociception by acting at spinal cord, brainstem or thalamic levels. Several lines of evidence suggest that 5-HT may regulate acetylcholine and noradrenaline release in the central nervous system (Sawynok and Reid, 1991; Fuerstein et al., 1996; Ramirez et al., 1997). 5-HT mediated spinal antinociception has also been shown to involve μ-opioid receptors (Goodchild et al., 1997). Recently, we reported the involvement of 5-$HT_1$ and 5-$HT_2$ receptors in fluoxetine-induced antinociception in diabetic mice (Anjaneyulu and Chopra, 2004), but the mechanism of indirect modulation of fluoxetine analgesia by other neurotransmitters needs to be evaluated in the diabetic pain.

Against this background, the present study aimed to investigate the possible involvement of cholinergic, adrenergic and opioid receptors with respect to the antinociceptive action of fluoxetine in streptozotocin-induced diabetic mice.

## 2. Material and methods

### 2.1. Animals

Male albino mice of Laka strain (20–30 g) bred in Central Animal House facility of Panjab University were used in the present study. The animals were housed under optimal laboratory conditions, maintained on a natural light and dark cycle, and had free access to food and water ad libitum. Animals were acclimatized to laboratory conditions before the test. All experiments were carried out blindly between 09:00 and 17:00 h. The experimental protocols were approved by the Institutional Animal Ethics Committee and conducted according to the Indian National Science Academy Guidelines for the use and care of animals (licence number: 388/Ethic/2002).

### 2.2. Drugs and reagents

Streptozotocin, atropine, naloxone and yohimbine were purchased from Sigma Chemicals (St. Louis, MO, USA). Fluoxetine (gift sample from Divis Pharma, India), atropine, naloxone and yohimbine were dissolved in distilled water. The glucose oxidase peroxidase enzyme kit was purchased from Span Diagnostics, India.

### 2.3. Induction and assessment of diabetes

Streptozotocin was prepared in citrate buffer (pH 4.4, 0.1 M) (Banisinath et al., 1988) and injected intraperitoneally in a single dose of 200 mg/kg (Ramabadran et al., 1989). The age-matched control mice received an equivalent volume of citrate buffer. At 2 days after streptozotocin injection, plasma glucose levels were estimated with the glucose oxidase peroxidase enzyme kit method (Schmidt, 1971). Plasma glucose levels were also measured at the time of 4 weeks. About 90% of streptozotocin-injected mice developed diabetes and mice with plasma glucose levels of more than 250 mg/dl (Anjaneyulu and Ramarao, 2002) were considered as diabetic and used for the present study after 4 weeks.

### 2.4. Treatment schedule

Preliminary threshold to tail-immersion and hot-plate responses (the means of two consecutive stable values which do not differ more than 10%) were determined before the drug administration. At the end of 4 weeks, control and diabetic mice were randomly divided into different groups consisting of 6–7 animals. Fluoxetine (10 and 20 mg/kg) was administered intraperitoneally in two groups of diabetic mice after measurement of baseline response. To other groups of diabetic mice, atropine (2 and 5 mg/kg), yohimbine (2 and 5 mg/kg) and naloxone (2 and 5 mg/kg) were injected intraperitoneally 5 min before the fluoxetine (20 mg/kg) injection. In both tail-immersion and hot-plate assays, nociceptive latency was measured at 15, 30, 60 and 180 min after fluoxetine injection and expressed as % of the maximum possible effect (% MPE), where % MPE = (post-drug threshold − pre-drug threshold) × 100/(cut-off time − pre-drug threshold). All drug solutions were freshly prepared and injected intraperitoneally in a constant volume of 1 ml/100 g of body weight.

### 2.5. Assessment of thermal hyperalgesia

#### 2.5.1. Tail-immersion (warm water) test

The tail was immersed in a warm water bath (52.5 ± 0.5 °C) until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 12 s). The hyperalgesic response in the tail-withdrawal test is generally attributed to central mechanisms (Ramabadran et al., 1989; Kannan et al., 1996).

#### 2.5.2. Hot-plate test

The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms (Kannan et al., 1996). In this test, animals were individually placed on a hot-plate (Eddy's Hot-Plate) with the temperature adjusted to 55 ± 1 °C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 10 s in order to avoid damage to the paw.

### 2.6. Statistical analysis

The nociceptive threshold, i.e., the latency (in seconds) to thermal noxious stimulus was measured and % MPE was calculated. The data are expressed as means ± S.E.M. The hyperalgesic response was analysed by analysis of variance followed by Tukey's *t*-test. Student's unpaired *t*-test was used to compare the values from two groups. $P < 0.05$ was considered as significant.

## 3. Results

### 3.1. Effect of streptozotocin injection in mice on blood glucose and body weight

Four weeks after streptozotocin injection, the diabetic mice had significantly higher blood glucose levels (486.44 ± 25.58 mg/dl) than the control mice (106.92 ± 18.42 mg/dl; $P < 0.001$). There

Table 1
General parameters in STZ-induced diabetic mice

| | Basal | 1 week | 2 weeks | 3 weeks | 4 weeks |
|---|---|---|---|---|---|
| Body weight (g) | 27±1.46 | 22.4±1.41[a] | 19±1.54[a] | 17.2±1.50[a] | 16.2±1.65[a] |
| Tail-immersion (s) | 5.23±0.95 | 2.86±0.9[a] | 1.86±0.32[a] | 1.62±0.35[a] | 1.46±0.25[a] |
| Hot-plate (s) | 4.64±0.89 | 3.82±0.24 | 2.83±0.32[a] | 1.54±0.8[a] | 1.34±0.23[a] |

Data are means±S.E.M. [a]$P<0.001$ as compared with its basal.

was a marked decrease in the body weight of streptozotocin-injected mice (16.8±2.10 g) as compared with control mice (29.24±5.48 g; $P<0.001$).

### 3.2. Effect of streptozotocin injection on nociceptive threshold

The nociceptive threshold was significantly lower in diabetic mice as compared with the basal value tested in both the tail-immersion and hot-plate assays. Hyperalgesia was evident in the tail-immersion and hot-plate after 1 week and 2 weeks respectively, and the maximum decrease in pain threshold was observed at 4 weeks after streptozotocin injection in mice (Table 1).

### 3.3. Effect of fluoxetine on nociceptive threshold in control and streptozotocin-induced diabetic mice

Systemic administration of fluoxetine (10 and 20 mg/kg) produced a significant increase in % MPE in both the tail-immersion and hot-plate (Fig. 1, upper and lower panels respectively) assays as compared to untreated diabetic mice. The % MPE produced by fluoxetine (20 mg/kg) was significantly lower in diabetic mice than in the control mice. The maximum % MPE was observed at 60 min after administration of fluoxetine.

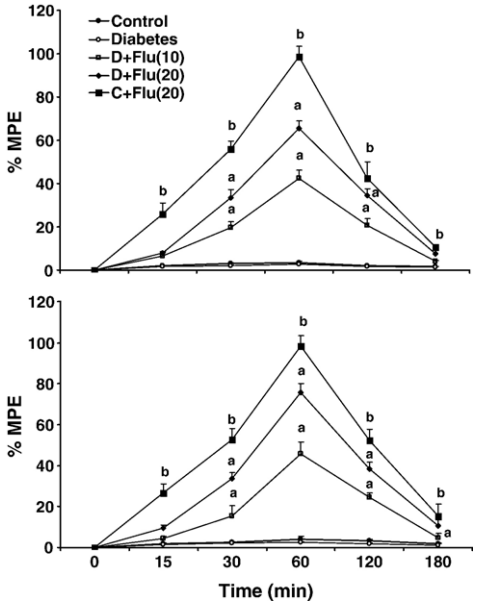


Fig. 1. Effect of fluoxetine on tail-immersion (upper panel) and hot-plate (lower panel) nociceptive threshold in control and streptozotocin-induced diabetic mice. FLU(10): fluoxetine (10 mg/kg); FLU(20): fluoxetine (20 mg/kg). Data expressed as mean±S.E.M ($n=7$ in each group). [a]$P<0.001$ as compared with diabetic mice at the respective times; [b]$P<0.001$ as compared with control mice at the respective times.

### 3.4. Effect of atropine, naloxone and yohimbine on antinociceptive action of fluoxetine in streptozotocin-induced diabetic mice

Prior administration of atropine (2 and 5 mg/kg) and naloxone (2 and 5 mg/kg) administration before fluoxetine (20 mg/kg) in diabetic mice resulted in significant attenuation of the % MPE of fluoxetine in both the tail-immersion and hot-plate systems. Pretreatment with yohimbine (2 and 5 mg/kg) did not alter the % MPE of fluoxetine in diabetic mice in both the tail-immersion and hot-plate (Fig. 2, upper and lower panels respectively) assay systems.

## 4. Discussion

In the present study, mice injected with streptozotocin exhibited significantly increased plasma glucose levels, urine output and decrease in body weight as compared with control mice. In diabetic mice, the tail-immersion latency was significantly shorter than that in non-diabetic mice, indicating

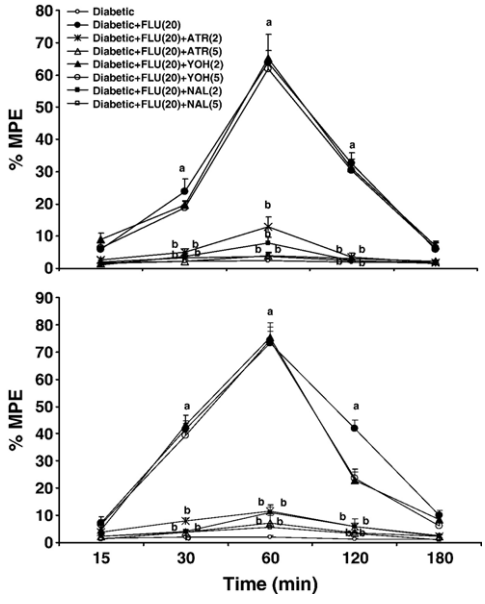


Fig. 2. Effect of fluoxetine and its combination with atropine, yohimbine and naloxone on tail-immersion (upper panel) and hot-plate (lower panel) nociceptive threshold in streptozotocin-induced diabetic mice. FLU(10): fluoxetine (10 mg/kg); ATR(2): atropine (2 mg/kg); ATR(5): atropine (5 mg/kg); YOH(2): yohimbine (2 mg/kg); YOH(5): yohimbine (5 mg/kg); NAL(2): naloxone (2 mg/kg); NAL(5): naloxone (2 mg/kg). Data expressed as mean±S.E.M ($n=6$ in each group). [a]$P<0.001$ as compared with diabetic mice. [b]$P<0.001$ as compared with diabetic+fluoxetine (20) mice at respective times.

that diabetic mice exhibit thermal hyperalgesia. This is in line with the observation of Ohsawa and Kamei (1999) demonstrating that streptozotocin-injected diabetic mice exhibited thermal allodynia and hyperalgesia, tested by exposing the tail to noxious stimuli.

Streptozotocin-induced diabetic mice have been used as a model of chronic pain with signs of hyperalgesia and allodynia that may reflect symptoms observed in diabetic humans (Gul et al., 2000; Kamei et al., 2001). The altered pattern of nociception may not be due to the inherent neurotoxicity of streptozotocin (Calcutt and Chaplan, 1997), but streptozotocin may induce a variety of pathophysiological symptoms that can lead to altered nociceptive responses tested in various animal models (Hounsom and Tomlinson, 1997; Kamei et al., 2001).

In the present study, fluoxetine produced a marked dose-dependent antinociception in control and streptozotocin-induced diabetic mice tested in the both tail-immersion and hot-plate assays. These results support earlier findings showing that systemic administration of fluoxetine produces antinociception in spinal nerve ligation, inflammation and other pain models (Filho and Takashi, 1999, Singh et al., 2001). In a double-blind randomized-controlled trial, it is also reported that fluoxetine act as co-analgesic (Vahedi et al., 2005). The mechanisms of action of antidepressant-induced antinociception remain unclear; however, it is well known that the reuptake of monoamines is a major mechanism of their pharmacological action (Hyttel, 1994). The attenuation of diabetic hyperalgesia by fluoxetine in the tail-immersion and hot-plate systems indicates an important role of serotonergic modulation in the nociceptive response, because it is well documented that serotonergic antidepressants, such as fluoxetine, increase synaptic 5-HT levels. In our previous study, we found that fluoxetine induces the antinociception in the diabetic mice through participation of 5-$HT_1$ and 5-$HT_2$ receptors (Anjaneyulu and Chopra, 2004).

The present study was an attempt to explore whether fluoxetine-induced antinociception is a direct effect of modulation of the 5-HT system or it is due to interplay between 5-HT and opioidergic, cholinergic and adrenergic system(s). Pretreatment with atropine and naloxone but not with yohimbine dose-dependently inhibited the antinociceptive activity of fluoxetine in both test systems of diabetic mice. Attenuation of antinociceptive action of fluoxetine by atropine in diabetic mice indicates that muscarinic receptors play an important role in the modulation of pain perception by serotonin. This is in support with other reports showing that intrathecal administration of atropine prior to 5-$HT_2$ agonist significantly attenuated the anti-allodynic effect in nerve ligation model (Obata et al., 2001). Further, intraperitoneal administration of atropine is reported to block the antinociceptive action of 5-$HT_1$ receptor agonist tested in the hot-plate method (Galeotti et al., 1997). One possible mechanism may include modulation of acetylcholine release from cholinergic interneurons in the spinal and supraspinal sites in the central nervous system (Fuerstein et al., 1996; Ramirez et al., 1997). It is reported that activation of somadendritically located 5-$HT_{2A}$ receptors facilitates release of substance P, which in turn stimulates acetylcholine release through tachykinin $NK_1$ receptors located in cholinergic terminals. Ramirez et al. (1997) reported that not only 5-$HT_{2A}$ receptor antagonists but tachykinin $NK_1$ receptor antagonists could also reduce acetylcholine release induced by dopamine $D_1$ receptors stimulation. In one of our earlier studies, fluoxetine-induced antinociception in diabetic mice was blocked by 5-$HT_2$ receptor antagonist ritanserin (Anjaneyulu and Chopra, 2004). Thus systemically administered fluoxetine by acting at 5-$HT_{2A}$ receptors may cause release of substance P which in turn would act at tachykinin $NK_1$ receptors to cause acetylcholine release.

Pretreatment with yohimbine, an $\alpha_2$-adrenoceceptor antagonist, did not affect the fluoxetine-induced antinociception in both tail-immersion and hot-plate assays. Intrathecal administration of yohimbine did not produce any antinociceptive effect in the transcutaneous electrical nerve stimulation in rats (Radhakrishnan et al., 2003). It is also observed that pretreatment with yohimbine does not attenuate the anti-allodynic action of intrathecal administration of 5-$HT_{2A}$ agonist in nerve ligation model (Obata et al., 2001). These results are conflicting with reports demonstrating interactions with noradrenaline in spinal antinociception mediated by 5-HT (Sawynok et al., 1991). The reason for this controversy is not fully understood, however the analgesic effect of 5-HT was dependent on the release of endogenous noradrenaline, at 5-$HT_1$ receptors while this was not true for the effect at 5-$HT_2$ receptor (Sawynok and Reid, 1992; Sawynok and Reid, 1996).

Pretreatment with naloxone significantly attenuated the antinociceptive action of fluoxetine in both tail-immersion and hot-plate assays in diabetic mice. This is further confirmed by Singh et al. (2001), where the antinociceptive action of fluoxetine was blocked by naloxone in naïve animals. It is also suggested that fluoxetine alone or co-administered with either imipramine or melatonin would be of benefit in the sitting of neuropathic or inflammatory pain conditions. Both the serotonergic and the opioid systems are likely to be involved in the modulating action of fluoxetine on peripheral inflammation (Abdel-Salam et al., 2004). Recently, it has been also reported that the reduced antinociceptive effect of morphine in diabetes is due to the decreased synaptic levels of 5-HT (Sounvoravong et al., 2004) and serotonin precursor can be responsible for restoration of antinociceptive action of morphine (Nemmani and Mogil, 2003). In a number of studies, a direct role of the opioidergic system in antidepressants-induced antinociception is reported (Botney and Fields, 1983; Auerbach et al., 1985; Sounvoravong et al., 2004). Similarly, in the present study fluoxetine-induced antinociception may also involve an interaction with opioid receptors in diabetic condition by altering the synaptic 5-HT levels in the brain.

In conclusion, the antinociceptive effect of systemically administered fluoxetine involves primarily the involvement of the serotonergic pathway and resultant increase in 5-HT levels may indirectly modulate the muscarinic and opioidergic, but not the adrenergic pathways in diabetic neuropathic pain.

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