Experimental Investigations

EXPERIMENTAL STUDY ON THE ROLE OF 5-HT\textsubscript{2} SEROTONIN RECEPTORS IN THE MECHANISM OF ANTI-INFLAMMATORY AND ANTIHYPERALGESIC ACTION OF ANTIDEPRESSANT FLUOXETINE

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\textbf{ABSTRACT}

\textbf{INTRODUCTION:} Fluoxetine is an antidepressant that has anti-inflammatory and antihyperalgesic effects in experimental models of pain and inflammation. The aim of the present study was to determine the role of 5-HT\textsubscript{2} receptors in the mechanism of anti-inflammatory and antihyperalgesic action of fluoxetine after single and repeated administration of the drug. \textbf{MATERIALS AND METHODS:} 40 male Wistar rats were randomly divided in five groups (n = 8) treated for 14 days with saline (control), diclofenac (positive control), fluoxetine, cyproheptadine (5-HT\textsubscript{2} antagonist), and fluoxetine + cyproheptadine, respectively. We used the experimental model of inflammation induced by intraplantar injection of carrageenan and nociceptive test with mechanical pressure on the inflamed hind paw. \textbf{RESULTS:} Single and repeated administration of fluoxetine showed that it had significant anti-inflammatory and antihyperalgesic effects when compared with the control (p < 0.05). Cyproheptadine did not change significantly the anti-inflammatory effect of fluoxetine in the first 4 hours, after a single administration. At 24 hours the combination did not differ statistically when compared with the control. Cyproheptadine did not change significantly the anti-inflammatory effect of fluoxetine after repeated administration. After prolonged treatment the group that received fluoxetine + cyproheptadine showed a statistically significant increase in paw pressure to withdraw the hind paw compared with that treated with fluoxetine alone (p < 0.05). \textbf{CONCLUSIONS:} Fluoxetine has anti-inflammatory and antihyperalgesic effects in the carrageenan model of inflammation. 5-HT\textsubscript{2} receptor mediated its anti-inflammatory effect in single dose treated animals. Spinal 5-HT\textsubscript{2} receptors are involved in the antihyperalgesic effect of fluoxetine after repeated administration.

\textbf{Key words:} fluoxetine, carrageenan, inflammation, antihyperalgesia, 5-HT\textsubscript{2} receptors

\textbf{ПЕРЕЧИСЛЕНИЕ}

\textbf{Введение:} Флуоксетин представляет собой антидепрессант, обладающий противовоспалительным и антигиперальгезическим эффектами при экспериментальных моделях боли и воспаления. Цель: Настоящее исследование ставит себе целью установить роль 5-HT\textsubscript{2} рецепторов в механизме противовоспалительного и антигиперальгезического действий флуоксетина при однократном и многократном применении. \textbf{Материал и методы:} На 40 мужских крыс породы „Вистар”, разделенных на 5 групп (n = 8), в течение 14 дней воздействовали физиологическим раствором (контрольная группа); диклофенаком (позитивная контрольная группа); флуоксетином, ципрохентадином (5-HT\textsubscript{2} антагонист) и флуоксетином + ципрохентадином. Использованы модель воспаления (интрапланетарное введение карагенина) и ночные тестовый (механическое давление на воспаленную лапку). \textbf{Результаты:} При однократном и многократном применении флуоксетина наблюдались сигнификантный противовоспалительный
INTRODUCTION

Fluoxetine is an antidepressant of the group of selective serotonin reuptake inhibitors (SSRI). It blocks serotonin transporter protein by high-affinity mechanism and increases the concentration of this mediator in the central nervous system (CNS) and peripheral tissues.\(^1\) It has been demonstrated that in addition to its main pharmacological antidepressive effect, fluoxetine has an anti-inflammatory activity.\(^2\) Studies on the anti-inflammatory activity of antidepressants are of interest in several areas. According to the neuroinflammatory hypothesis depressive disorders are related with inflammation in some brain structures.\(^3\) This raises the question about the possible involvement of the anti-inflammatory effect of antidepressants in their therapeutic efficacy in depression. On the other hand the anti-inflammatory effect of antidepressants may be useful in the treatment of inflammatory diseases that accompany depression or antidepressants can be used alone for this purpose. For example, there is clinical evidence that some antidepressants, such as bupropion can induce remission in Crohn’s disease, psoriasis and atopic dermatitis.\(^4\)

Fluoxetine has analgesic activity in different experimental models of pain and antinoceptive tests. Singh et al. found that this antidepressant has pronounced analgesic effect in three classic pain tests – the tail-flick, hot plate and abdominal constrictor tests. They observed this effect after systemic and intracerebroventricular administration of fluoxetine.\(^5\) Furthermore, fluoxetine delays development of tolerance to morphine analgesia.\(^6\) In genetically modified mice lacking central serotonergic neurons (line Lmx1b\(^{f/f,p}\)) the analgesic action of fluoxetine is completely absent in pain tests using thermal stimulus, mechanical pressure of inflamed paw and formalin test. In wild type mice systemic injection of fluoxetine elicits a strong analgesic effect in the same pain models excluding the first phase of the formalin test.\(^7\)

The mechanism of the analgesic and anti-inflammatory effect of fluoxetine is not well understood. It increases the levels of serotonin which in turn acts on a number of receptor subtypes expressed in the CNS and peripheral tissues. There is evidence that suggests that 5-HT\(_2\) receptors induce spontaneous pain and hyperalgesia in the peripheral tissues where they mediate pro-inflammatory effects of serotonin.\(^8\) In contrast to the periphery in the CNS 5-HT\(_2\) receptors probably mediate ant-inflammatory and analgesic effect.

Carrageenan-induced paw edema is a frequently used experimental model of inflammation. The tricyclic antidepressants amitriptyline, imipramine and clomipramine exhibit significant anti-inflammatory effect in this model.\(^9\)

AIM

The aim of the present study was to determine the role of 5-HT\(_2\) receptors in the mechanism of anti-inflammatory and antihyperalgesic action of fluoxetine after single and repeated administration of the drug.

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee on Animal of the Bulgarian Food Safety Agency with permit No 56/19.03.2012 and with a decision of the Ethics Committee at the Plovdiv Medical University, No 4/19.06.2013.
ANIMALS
Male Wistar rats with mean weight of 220 – 250 g were used. Animals were randomly allocated into five groups (n = 8) treated for 14 days as follows: Group 1 (controls) – control group treated with saline intraperitoneally (i. p.); Group 2 (positive control group) - treated with a reference anti-inflammatory and analgesic drug diclofenac in a dose of 25 mg/kg bw (i. p.) ; Group 3 - treated with fluoxetine in a dose of 20 mg/kg bw (i. p.); Group 4 - treated with 5-HT₂ receptor antagonist cyproheptadine in a dose of 5 mg/kg bw (i. p.); Group 5 - treated with fluoxetine 20 mg/kg bw (i. p.) and cyproheptadine 5 mg/kg bw (i. p.).

The animals were housed under standard laboratory conditions: 12:12 h light/dark cycle, 45% relative humidity, room temperature 26.5 ± 1 °C and free access to food and tap-water. The experiments were conducted between 8:00 a.m. and 3:00 p.m. All groups were tested for analgesic and anti-inflammatory activity after a single and repeated (14 days) treatment. For the groups treated with the receptor antagonist the latter was applied only on the day of the experiments.

EXPERIMENTAL METHODS
Carrageenan-induced paw edema.

The carrageenan-induced hind paw swelling was measured using a plethysmometer (Ugo Basile, Italy). Prior to treatment the volume of the right hind paw of the animals from all groups was measured. Thereafter all animals were given an intraplantar injection of 0.1 ml of 1% solution of carrageenan in 0.9% sodium chloride to induce an inflammatory edema. Immediately after the injection of carrageenan animals from control group received 0.1 ml/100 g bw saline; the positive control group was treated with diclofenac sodium (25 mg/kg bw) and the animals in the experimental groups were injected the test substances i. p. In the groups treated with more than one substance the second substance was administered 30 minutes after the first one. Hind paw volume was measured immediately before carrageenan injection and at 2, 3, 4 and 24 hours thereafter with a plethysmometer. The percentage of paw edema was calculated using the following equation:

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Paw\ \text{edema}\ (%) = \frac{(V - V_0)}{V_0} \times 100,
\]

where V is the paw volume at 2, 3, 4 and 24 hours after carrageenan injection and V₀ is the paw volume prior to carrageenan injection.

The animals were tested for anti-inflammatory action on days 1 and 14 of treatment.

Nociceptive test with mechanical pressure of carrageenan-inflamed paw (analgesimeter).

The test was described by Randall & Selitto (1957). Analgesimeter (Ugo Basile, Italy) is used in the test. This method is used for rapid precise screening of analgesic drugs. Mechanical pain stimulus is applied on a normal paw or an inflamed rat paw. Nociceptive threshold is measured by applying pressure on the inflamed rat hind paw. The strength of the pressure at which the animal withdraws testing paw is recorded. The maximal possible pressure is 250 grams (cut off). The rats were tested one hour before the treatment and at 1, 2 and 3 hours after the treatment. For groups treated with two drugs rats were tested at 1, 2 and 3 hours after the treatment with the second drug. The pressure at which the animal withdraws the hind paw was expressed as % of the maximum possible effect (MPE %), where MPE % = (post-treatment threshold – pretreatment threshold) x 100/(250 – pretreatment threshold).

STATISTICAL ANALYSIS

Data were analyzed using the analysis of variance - One Way Anova from the software product SPSS 11.0. Mean values ± SEM were calculated. Nonparametric test of Kolmogorov-Smirnov show a normal distribution. A comparison of the results between groups was done using Independent – Samples T test. The results were considered significant at p < 0.05.

RESULTS
Effect of cyproheptadine on the anti-inflammatory action of fluoxetine in carrageenan-induced inflammatory reaction.

A single administration of fluoxetine significantly reduced carrageenan-induced paw edema in the early phase of carrageenan inflammation (p = 0.04 at 2 hours and p = 0.01 at 4 hours) and at 24 hours (p < 0.0001) when compared with control. There was no statistically significant difference between anti-inflammatory effect of fluoxetine and that of diclofenac, used as a reference anti-inflammatory drug. Cyproheptadine reliably inhibited the early and late phase of carrageenan edema in comparison with the control. Co-administration of fluoxetine (20 mg/kg bw) and cyproheptadine (5 mg/kg bw) did not change statistically significantly the effect of fluoxetine on the inhibition of carrageenan-induced edema in single dose treated animals in the first 4 hours. Although not significant the effect of the
combination was more pronounced than that of the two substances administered separately. At 24 hours the combination did not differ significantly when compared with the control but fluoxetine (p < 0.0001) and cyproheptadine (p < 0.0001) alone significantly reduced the carrageenan-induced inflammatory edema (Fig. 1).

After repeated treatment (14 day) fluoxetine significantly reduced carrageenan-induced edema at 2, 3, 4 hours (p < 0.0001 ) and at 24 hours (p = 0.01) when compared with the control. The anti-inflammatory effect of fluoxetine was significantly lower than that of diclofenac only at 24 hour (p < 0.05). Cyproheptadine statistically significantly inhibited the early and late stages of the carrageenan edema in comparison with the control, but did not alter significantly the anti-inflammatory effect of fluoxetine. At 24 hours the extent of reduction of the edema from the combination (36.22%), although not statistically significant, was more pronounced than that of fluoxetine (49.11%), and cyproheptadine (52.98 %) administered alone (Fig. 2).

Effect of cyproheptadine on the antihyperalgesic action of fluoxetine in the test with mechanical pressure of inflamed rat hind paw.

Single administration of fluoxetine in a dose of 20 mg/kg bw significantly increased the value of MPE % at 1 hour (p = 0.004), 2 hours (p = 0.01) and 3 hours (p = 0.002) when compared with the controls. Diclofenac used as a substance with known anti-inflammatory and analgesic effect increased significantly the strength of the pressure for paw withdrawal at the three tested hours (p = 0.005, p < 0.0001 and p = 0.001, respectively) when compared with the controls (Fig. 3).

In the group with repeated administration of diclofenac we observed a significant increase of MPE % at 2 hours (p = 0.01) and at 3 hours (p = 0.04) (in comparison with the controls). The group treated for 14 days with fluoxetine in a dose of 20 mg/kg bw showed a significant increase in the values of MPE % at 1 hour (p = 0.004), 2 hours (p = 0.003) and 3 hours (p < 0.0001 ) (Fig. 4).

After single application, cyproheptadine did not change statistically significantly the effect of fluoxetine in the test with mechanical pressure of inflamed rat hind paw. In the group with co-administration of cyproheptadine and fluoxetine we found a non-significant decrease of MPE% at 2 and 3 hours, and at 3 hours the group treated with both agents did not differ statistically from the control.

After repeated administration the group that received fluoxetine and cyproheptadine differed significantly (decrease in MPE% was observed) from that treated with fluoxetine alone (p = 0.03) (Figs 3, 4).

**DISCUSSION**

The results in our study indicate that SSRI anti-
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**Figure 3.** Effect of 5-HT₂ receptor antagonist cyproheptadine on the antihyperalgesic effect of fluoxetine in carrageenan model of inflammation and single dose treatment.

**Figure 4.** Effect of 5-HT₂ receptor antagonist cyproheptadine on the antihyperalgesic effect of fluoxetine in carrageenan model of inflammation after repeated treatment.

The antidepressant fluoxetine has anti-inflammatory and antihyperalgesic activity in carrageenan-induced inflammation. This effect was detected in the early and late phases of inflammation after single and repeated administration. Our results are in agreement with those of Abdel-Salam et al. for anti-inflammatory activity of fluoxetine in this model of inflammation.²

In the CNS, the 5-HT₂ receptors mediate anti-inflammatory effect. In experimental conditions intracerebroventricular injection of exogenic 5-HT on rats with normal serotonin levels reduces carrageenan edema.¹⁰ 5-HT₂ receptors interact with 5-HT₃ receptors in CNS which in turn stimulate serotonin secretion. Cyproheptadine as antagonist of 5-HT₃ receptors should inhibit the observed anti-inflammatory effect of fluoxetine if it is realized by a central mechanism. In the present study cyproheptadine blocks this effect only 24 hours after single administration. The anti-inflammatory effect of serotonin in the CNS can be explained with its neuroendocrine action. In situ hybridization on rat brain slices with oligopeptides showed an increase of corticotropin releasing hormone mRNA in the paraventricular nucleus and proopiomelanocortin in the anterior pituitary lobe upon stimulation of 5-HT₂a and 5-HT₂b receptors. The stimulation of ACTH secretion and hence of hydrocortisone from the adrenal glands is also mediated through the 5-HT₂ receptors.¹¹ Our data show that these central mechanisms play a limited role in the observed anti-inflammatory effect of fluoxetine and perhaps the increased concentration of serotonin in the brain is not the main mechanism to achieve this effect.

5-HT receptors expressed in the peripheral tissues mediate pro-inflammatory effect of serotonin. Carrageenan-induced inflammatory reaction through 5-HT₂ receptors stimulates the expression of the receptors for the calcitonin-gene related peptide (CGRP). CGRP that is released from both the peripheral and central terminals of primary afferent fibers during the inflammation leads to sensitization of nociceptors, nociceptive neurons in the spinal cord and dorsal root ganglia. Suplantar injection of a 5-HT₂ receptor antagonist ketanserine suppresses the increased expression of CGRP and inhibits hyperalgesia.¹² On the other hand the inhibition of these neurons by 5-HT₂ antagonists is not direct but is mediated by opioidergic mechanisms. 5-HT suppressed migration of inflammation-activated β-endorphin-containing cells towards the site of inflammation. This is probably realized via 5-HT₂ receptors because their antagonists such as ketanserin increase the number of these cells in the inflammation. Cyproheptadine as a 5-HT₂ receptor antagonist, probably by blocking those receptors, enhances endogenous opioid mechanisms and inhibits CGRP expression in the spinal cord induced by carrageenan inflammation. This explains the observed anti-inflammatory effect in the experimental model of inflammation with carrageenan. This ef-
flect occurs in both single and repeated (14 days) administration. The effect is comparable to that of fluoxetine. Co-administration of fluoxetine and cyproheptadine did not alter the anti-inflammatory effect of the antidepressant with the exception of the 24th hour after single dose treatment. The observed additive synergism can be attributed to the poor affinity of the fluoxetine for the 5-HT	extsubscript{2} receptors and its ability to block them. Studies have demonstrated the interesting fact that both the stimulation of these receptors (from agonists or SSRI) and their long-time blockade lead to down regulation.\textsuperscript{13} These facts explain the lack of even transient pro-inflammatory effect of fluoxetine in the present study due to increased serotonin levels in the periphery.

In the experimental model of inflammatory hyperalgesia cyproheptadine significantly decreased the effect of fluoxetine at 3 hours in continuously treated animals. Since behavioral responses in this test are mediated by centers in the spinal cord\textsuperscript{14} we can assume that spinal 5-HT	extsubscript{2} receptors are essential in the mechanism of the anti-hyperalgesic effect of fluoxetine after multiple dosing. Intratecal administration of 5-HT	extsubscript{2A/2C} receptor agonist \(\alpha\)-methyl-5-hydroxytriptamine has an antinoceptive effect in the formalin test and chronic sciatic nerve injury.\textsuperscript{15} A possible explanation for the antihyperalgesic effect of 5-HT	extsubscript{2} receptors in the spinal cord is that they stimulate the release of inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) from the interneurons in the spinal dorsal horn. There are studies which have shown that about 90\% 5-HT	extsubscript{2A} positive cells in the periaqueductal gray matter of the rat are immunoreactive for GABA.\textsuperscript{16} It has been demonstrated using molecular genetic techniques that a small percentage (about 10\%) of the cells in the rat spinal dorsal horn express mRNA for these receptors.\textsuperscript{17} This location would mediate an antinociceptive effect due to induction of the GABA/glycinergic inhibitory potentials in spinal cord via 5-HT	extsubscript{2} receptor stimulation.\textsuperscript{18} The weak role of these receptors in the analgesic action of fluoxetine in the test with mechanical pressure of inflamed rat hind paw is probably due to the expression of inhibitory proteins – postsynaptic density protein. It has been found that administration of fluoxetine with inhibitors of this protein enhances the analgesic effect mediated by 5-HT	extsubscript{2} receptors.\textsuperscript{19}

**CONCLUSIONS**

Fluoxetine has anti-inflammatory and antihyperalgesic effects in carrageenan model of inflammation. Centrally localized 5-HT	extsubscript{2} receptor mediated its anti-inflammatory effect only in single dose treated animals in the late phase of carrageenan inflammation. Spinal 5-HT	extsubscript{2} receptors are involved in the antihyperalgesic effect of fluoxetine after repeated administration.

**REFERENCES**

Experimental Study on the Role of 5-HT$_2$ Serotonin Receptors in the Mechanism of Anti-inflammatory and Antihyperalgesic Action of Antidepressant Fluoxetine