Preclinical evaluation of the impact of cilostazol on anti-depressant activity of fluoxetine

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ABSTRACT
The current anti-depressant agents have limitations like the slow onset of action, moderate efficacy, withdrawal symptoms, incompliance of treatment, and instabilities in circadian rhythm. Their therapeutic use is quite restricted and produces inadequate or partial symptomatic relief of depression which may lead to treatment- resistance depression. In the view of a new strategy, second messengers (cAMP, cGMP) and their signalling pathways are emerging as novel targets for anti-depressants. The present study conducted to evaluate the augmentation property of cilostazol on the anti-depressant activity of fluoxetine. Traditional anti-depressant models like forced swimming test and tail suspension test were employed. Mice were randomly grouped into six groups, with six rats in each. Each group was treated, as mentioned in the study. The reduction in immobility period of each mouse was noted. The results were analysed by ordinary one way ANOVA followed by Tukey's multiple comparison test. Cilostazol at a dose of 20 mg/kg i.p significantly reduced immobility period when compared to cilostazol 10 mg/kg i.p and normal saline. Cilostazol 10 mg/kg i.p also decreased immobility period significantly when compared to normal saline by forced swimming test. Fluoxetine 20 mg/kg i.p + cilostazol 20 mg/kg i.p produced a highly significant reduction in the immobility period in comparison with all groups except with fluoxetine 20 mg/kg i.p + cilostazol 10 mg/kg i.p in forced swimming test. This study concludes that cilostazol has produced dose-dependent anti-depressant activity. This study also emphasises cilostazol can augment the anti-depressant activity of fluoxetine.

INTRODUCTION
Depression is derived from a Latin word called depressio, which is regarded as sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness, and low concentration (Bernard, 2018). According to the World Health Organization, depression is a significant mental health disorder in population with irrespective of age, gender, and socio-economic status but the prevalence varies with region, age, gender, and socio-economic status. Prevalence is more in older adults (55-74 years)
than children and adolescents (<15 years). Three hundred twenty-two million peoples are living with depression which is about 4.4% of world inhabitants (Grover et al., 2019; World Health Organization, 2017).

Out of 4, three patients are not getting adequate treatment, and in severe conditions, depression can lead to suicide (World Health Organization, 2020). Approximately 8,00,000 persons die owing to suicide every year. Suicide is the 2nd leading cause of death in 15-29 years individuals (World Health Organization, 2020). Depressive disorders include two main sub-types: major depressive disorder / depressive episode and dysthymia (Marcus et al., 2012).

It is challenging to argue by focussing on one factor of depression since there are many diverse factors like biological, genetic, hormonal, family, environmental, and socio-economical factors (Bembrowska and Jośko-Ochojska, 2015). The currently available anti-depressant agents have limitations which may include the slow onset of action, moderate efficacy, withdrawal symptoms, incompliance of treatment, and instabilities in circadian rhythm (Lader, 2007). Their therapeutic use is quite restricted and produces inadequate or partial symptomatic relief of depression (Kautzky et al., 2019; Rush et al., 2003).

Minimum four anti-depressant treatment failures with or without electroconvulsive therapy (ECT) failure is defined as treatment-resistance depression (TRD) (Philip et al., 2010). The clinical strategy to treat TRD is an adjustment in a regimen that may include optimisation of dose, route, therapy course, augmentation/combination therapies, and interchanging therapies with the drugs of the same class or different group (Souery et al., 2006). Though mentioned, optimised or adjusted strategies are not enough to meet the pharmacological approach for TRD, augmentation/combination therapies are having better shreds of evidence. These include augmentation of SSRI’s anti-depressant activity with aripiprazole, lithium, triiodothyronine (T3) with limitations (Kim et al., 2017; Agid et al., 2001; Price et al., 2008).

Phosphodiesterases (PDEs), are a cluster of enzymes entailing 11 families with multiple isoforms in some families. The cyclic nucleotides have tremendous and vital pleiotropic effects on cell functions. These enzymes are distributed in different regions of the brain. So in the recent period alteration in cyclic nucleotides levels in the neuronal cells have got an attraction in the therapeutic research of neurological disorders (Hebb and Robertson, 2007). As cilostazol inhibits phosphodiesterase 3, there is an increase in levels of cAMP in the cell (Gresele et al., 2011).

Cilostazol is a thrombolytic agent, and a vasodilator approved as one of the therapeutic agents for intermittent claudication and prevention of ischemic stroke (Bedenis et al., 2014; Oyama et al., 2011). There are very few studies on the anti-depressant activity of cilostazol. Here the present study focused on anti-depressant activity with two log doses (10 mg/kg, 20 mg/kg), augmentation properties of cilostazol when combined with fluoxetine.

**MATERIALS AND METHODS**

**Animals**

25g -35g weighed Wister strain albino mice of both sexes were employed in the study. The mice were fed with water, adlibitum, and standard pellets in the animal house of Fathima Institute of Medical Sciences, Kadapa, in optimal laboratory conditions which includes 12 h light / 12 h dark cycle, the temperature was maintained at 25°C ± 1°C. Each animal was employed once in the experiment and followed CPCSEA guidelines. Exclusion criteria include any deviation in weight and signs of illness. The experimental study got approval from the local ethical committee (IAEC).

**Drugs**

The standard drug used was an analytical grade. Cilostazol was purchased from Pure Chem. Private Ltd was liquefied in DMSO, which was purchased from Pon Pure Chemicals (Gad et al., 2006).

**Animal grouping**

Group 1 - Normal saline

Group 2 - Fluoxetine (20 mg/kg; i.p)

Group 3 - Cilostazol (10 mg/kg i.p)

Group 4 - Cilostazol (20 mg/kg i.p)

Group 5 - Fluoxetine (20 mg/kg; i.p) + Cilostazol (10 mg/kg i.p)

Group 6 - Fluoxetine (20 mg/kg; i.p) + Cilostazol (20 mg/kg i.p)

**Forced swimming test in mice (FST)**

The forced swimming test or behavioural despair swim test has been used as a test for depression-like behaviour.

**Principle**

When an animal is forced to swim, it tries to escape by making meticulous movements. The animal surrenders to the situation and floats by making very little or no movements when it cannot escape.
Table 1: Effect of treatments on the forced swimming test induced immobility period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immobility period (Mean ± Standard deviations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>170.33 ± 2.16</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p)</td>
<td>92.5 ± 4.27</td>
</tr>
<tr>
<td>Cilostazol (10 mg/kg i.p)</td>
<td>163.33 ± 4.54 @</td>
</tr>
<tr>
<td>Cilostazol (20 mg/kg i.p)</td>
<td>111.5 ± 3.27 @ !</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p)</td>
<td>88 ± 3.03 @ #$</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)</td>
<td>82 ± 4.33 @ #$</td>
</tr>
</tbody>
</table>

n = 6; @=p<0.001, *=p<0.05 vs Normal saline; ! = P<0.001 vs Fluoxetine; $ = p<0.001 vs Cilostazol 10 mg/kg; # = p<0.001 vs cilostazol 20 mg/kg.

Table 2: Effect of treatments on the tail suspension test induced immobility period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immobility period (Mean ± Standard deviations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>229.33 ± 3.32</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p)</td>
<td>142.16 ± 5.52 @</td>
</tr>
<tr>
<td>Cilostazol (10 mg/kg i.p)</td>
<td>220.83 ± 7.33 !</td>
</tr>
<tr>
<td>Cilostazol (20 mg/kg i.p)</td>
<td>188.5 ± 6.59 @ !</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p)</td>
<td>134.66 ± 5.12 @$#</td>
</tr>
<tr>
<td>Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)</td>
<td>122.33 ± 4.96 @$#</td>
</tr>
</tbody>
</table>

n = 6; @=p<0.001 vs Normal saline; ! = P<0.001 vs Fluoxetine; $ = p<0.001 vs Cilostazol 10 mg/kg; # = p<0.001 vs cilostazol 20 mg/kg; *= p<0.01 vs Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p).

Figure 1: Confidence Intervals by Tukey’s multiple comparison test of Forced swimming test observations

from the glass chamber. Thus forced swimming test induced immobility is considered as a state of depression in mice. This test is susceptible to all main classes of anti-depressant drugs.

Procedure

Mice were forced to swim individually in the glass chamber (measuring 40cm, 26cm, 26cm length, breadth height respectively) containing 15 cm height of water at room temperature for 15 minutes. This was considered a “pre-test session”. After 24 hours, group 2 animals were treated with standard drug (Fluoxetine 20 m/kg i.p), group 3 & 4 were treated with Cilostazol 10 mg/kg i.p, 20 mg/kg i.p. and group 5 & 6 were treated with Fluoxetine
Tail suspension test (TST)

Principle

When an animal is suspended upside down leads to a characteristic behaviour of immobility after an initial fight back to escape from the situation. This behaviour reflects a state of depression which can be reduced by anti-depressant drugs.

Procedure

Mice were suspended on the edge of a table 50 cm above from the floor by using adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during 6 min of the test. All randomly grouped animals were treated with respective drugs and dosage, as mentioned in the grouping of animals. After 30 minutes of drug administration, mice were suspended, and their immobile period was noted (Gupta, 2011; Kulkarni, 1987).

Statistical analysis

Observed duration of immobility time of each mouse was tabulated, and their Mean ± Standard deviations were calculated. The hypothesis was statistically tested by ordinary one way ANOVA followed by Tukey’s multiple comparison test. P<0.05 was revealed as a statistically significant outcome.

RESULTS AND DISCUSSION

Forced swimming tests (FST)

In Figure 1, N S: Normal saline; Flu: Fluoxetine (20 mg/kg i.p) Cilo 10 mg: Cilostazol (10 mg/kg i.p) Cilo 20 mg: Cilostazol (20 mg/kg i.p) Flu + Cilo 10 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p) Flu + Cilo 20 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)

In Figure 2, N S: Normal saline; Flu: Fluoxetine (20 mg/kg i.p); Cilo 10 mg: Cilostazol (10 mg/kg i.p); Cilo 20 mg: Cilostazol (20 mg/kg i.p); Flu + Cilo 10 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p); Flu + Cilo 20 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)

In Figure 3, N S: Normal saline; Flu: Fluoxetine (20 mg/kg i.p); Cilo 10 mg: Cilostazol (10 mg/kg i.p); Cilo 20 mg: Cilostazol (20 mg/kg i.p); Flu + Cilo 10 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p); Flu + Cilo 20 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)

In Figure 4, N S: Normal saline; Flu: Fluoxetine (20 mg/kg i.p); Cilo 10 mg: Cilostazol (10 mg/kg i.p); Cilo 20 mg: Cilostazol (20 mg/kg i.p); Flu + Cilo 10 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (10 mg/kg i.p); Flu + Cilo 20 mg: Fluoxetine (20 mg/kg i.p) + Cilostazol (20 mg/kg i.p)

The descriptive statistics tabulated in the Table 1, exhibited a statistically significant difference by ordinary one way ANOVA with F = (5, 30); p < 0.001. With consideration of Table 1 the present study highlighted that cilostazol 20 mg/kg i.p displayed a significant variance with normal saline (p < 0.001) and cilostazol 10 mg/kg i.p (p < 0.001). The study also emphasised a significant difference betweencilostazol 10 mg/kg i.p and normal saline (p < 0.05). Fluoxetine exhibited a highly considerable significant difference with all other groups (p < 0.001) except fluoxetine 20 mg/kg i.p + cilostazol 10 mg/kg

Figure 3: Confidence Intervals by Tukey’s multiple comparison test of tail suspension test observations

Figure 4: Residuals of tail suspension test observations
reduction of immobility period by both tests. Still, there was observed reduction in the immobility period by both tests. That indicates a combination of cilostazol 10 mg/kg i.p + fluoxetine 20 mg/kg i.p was significantly greater (p<0.001) with all other groups (p=0.312). Combination of cilostazol 20 mg/kg i.p with fluoxetine 20 mg/kg i.p exhibited greater significant difference with all other groups except fluoxetine 20 mg/kg i.p + cilostazol 10 mg/kg i.p (p=0.084). Confidence Intervals by Tukey’s multiple comparison test and residuals of Forced swimming test observations were plotted in the Figure 1 and Figure 2 respectively.

Tail suspension test (TST)

The descriptive statistics tabulated in the Table 2, exhibited a statistical significant difference by ordinary one way ANOVA with F = (5, 30); p<0.001. This study emphasised according to the Table 2 that cilostazol 20 mg/kg i.p exhibited significant variance with cilostazol 10 mg/kg i.p (p <0.001) and normal saline (p <0.001). Cilostazol 10 mg/kg i.p did not exhibit significance difference with normal saline (p=0.124). Fluoxetine 20 gm/kg i.p exhibited greater significant difference (p<0.001) with all other groups except fluoxetine 20 mg/kg i.p + cilostazol 10 mg/kg i.p (p = 0.222). The decrease in immobility period of fluoxetine 20 mg/kg i.p + cilostazol 20 mg/kg i.p was significantly greater with all other groups (p<0.001) and fluoxetine 20 mg/kg i.p + cilostazol 10 mg/kg i.p (p=0.01) was statistical significant. Confidence Intervals by Tukey’s multiple comparison test and residuals of tail suspension test observations were plotted in the Figure 3 and Figure 4 respectively.

In the present experimental study, depression was established by inducing an immobile state by forced swimming test and tail suspension test. These are well established, reliable models to evaluate anti-depressant activity. A decrease in the immobility period was considered as anti-depressant activity (Can et al., 2011; Stukalin et al., 2020).

Cilostazol 10 mg/kg i.p produced statistically significant difference with normal saline in Forced swimming test but not in tail suspension test. Still, there was a general decrease in immobility period in both tests. Cilostazol 20 mg/kg i.p produced better results when compared to low cilostazol dose (10 mg/kg i.p). The study emphasised that standard drug fluoxetine was superior to cilostazol both doses. A combination of cilostazol 10 mg/kg with fluoxetine 20 mg/kg i.p did not reveal a statistically significant difference with fluoxetine alone though there was observed reduction in the immobility period by both tests. That indicates a combination of cilostazol at the dose of 10 mg/kg was not superior to fluoxetine alone. A combination of cilostazol 20 mg/kg with fluoxetine 20 mg/kg i.p was exhibited significant difference with all other groups in reduction of immobility period by both tests. Still, this combination was not statistically superior with cilostazol 10 mg/kg i.p + fluoxetine 20 mg/kg. So augmentation of cilostazol 20 mg/kg i.p with standard drug fluoxetine exhibited superior activity in a decrease in the immobility period.

Mild cases can be treated with a single anti-depressant, but moderate to severe cases are not. Only 50-65% of patients respond to the first line mono-therapy with anti-depressants. There is no ideal anti-depressant in the effective anti-depressants available in the market because they are chosen by considering the symptoms, side effects, presence of associated diseases, and initial response by any other anti-depressant. Fluoxetine is a better drug in the present scenario. So the therapy was shifted to combination therapy to treat moderate, severe, and treatment-resistance depression (Iyer and Khan, 2012). The need to develop a new strategy came into picture apart from traditional monoaminergic systems. Fluoxetine (SSRI) significantly decreases the immobility period in the FST and TST.

Cilostazol inhibits phosphodiesterase 3 and elevates cAMP and cGMP in the cells which trigger the intracellular activation of Protein Kinase A, cyclic adenosine monophosphate response element-binding protein (CREB)/ Brain-derived neurotrophic factor (BDNF) signalling pathways in the hippocampus to possess anti-depressant activity (Kim et al., 2016; Carlezonjr et al., 2005). Cilostazol promotes proliferation and maturation of neuronal precursor cells by the CREB signalling pathway. A role of the CREB pathway in depression also demonstrated by Kim et al. (2017) in their study conducted to evaluate the beneficial effects of a lower active dose of aripiprazole with sub active dose of cilostazol in post-stroke depression. Psychological stress may produce an imbalance between oxidants and antioxidants which may lead to an increase in free radical levels, lipid peroxidation, DNA damage, and death of cells. Cilostazol activates the Nrf2 pathway mediated redox defence system to prevent oxidative stress and re-establishes mitochondrial functions (Abuelez and Hendawy, 2018).

Patel et al. (2012) Patel et al. conducted a preclinical study to demonstrate the anti-depressant activity of cilostazol in comparison with fluoxetine. In their study cilostazol, sodium carboxymethyl cellulose administered for 15 days each and fluoxetine for seven days by employing forced swimming test and tails suspension test with mice. The study observations revealed an anti-depressant activity of cilostazol by reducing immobility period 70.66±2.27 in forced swimming test and 102±5.66 in tail sus-
pension test (Patel et al., 2012). In the present study fluoxetine, cilostazol in two different doses and, their combinations administered and demonstrated anti-depressant activity in the agreement of with study in reducing the immobility period in FST and TST (Table 1 and Table 2). Bhatt et al. (2011) supported the present study, a one-month follow-up assessment study that demonstrated the anti-depressant activity of cilostazol in cardiovascular disease patients through the Montgomery–Asberg depression rating scale (MADRS). The subjects were administered 50 mg/kg of cilostazol twice a day. Gamil et al. (2016) demonstrated a significant anti-depressant activity in rats by cilostazol, tramadol, and their simultaneous administration in rodents by employing FST and TST. Simultaneous administration of cilostazol and tramadol demonstrated a statistically significant difference over control, with 66% decreases in the immobility period. Takahashi et al. (2008) demonstrated a novel augmentation activity of cilostazol in geriatric patients in their case presentation. The persistent major depressive disorder with deep white matter hyperintensities patient was administered cilostazol along with traditional anti-depressants and evaluated a prospective augmentation property of cilostazol. Hence all the studies indicate that cilostazol has got significant anti-depressant activity. In our study, it was proved that cilostazol not only demonstrated anti-depressant activity but also it augmented the anti-depressant activity of fluoxetine.

CONCLUSION

The present preclinical study concludes that cilostazol has produced dose-dependent anti-depressant activity and emphasises cilostazol (20 mg/kg i.p) can augment the anti-depressant activity of fluoxetine. Cilostazol and fluoxetine augmentation may be the right choice for cardiovascular disease (as anti-platelet anti-platelet agent) patients with treatment-resistance depression (TRD). Further studies are needed to evaluate the clinical significance of the above combination.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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