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PITAVASTATIN AND GEMFIBROZIL AS DRUG REPOSITIONING CANDIDATES FOR ANXIOLYTIC ACTIVITY: AN ANIMAL STUDY BY USING ELEVATED PLUS MAZE

Short title: Anxiolytic effects of pitavastatin and gemfibrozil

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Anxiety is a common finding in most neurotic disorders and plays a remarkable role in their pathogenesis. [1] Before the 20th century, "anxiety neurosis" and "pantophobia" terms were used to diagnose general anxiety. [2] In most anxiety cases, a person feels worsened episodes of symptoms followed by symptom-free episodes. This condition is referred to as "remitting and relapsing conditions." [3] The exact cause of anxiety is still a topic of discussion; it may be due to inflection in the central nervous system. [4] Dyslipidemia is a solid soothsayer of persistent medical conditions like obesity that could elevate the risk of anxiety. [5] Several pathways, such as neurotransmitter balance, oxidative stress, and immuno-inflammatory processes associated with anxiety, are also connected with obesity. [6] Metabolites suggestive of bad metabolic health

might be represented as distant biomarkers for anxiety. [7] Due to a poor understanding of the molecular mechanisms, few treatment options are available to treat anxiety. [8] A multidisciplinary approach is selected nowadays to treat anxiety, such as psychotherapy and pharmacotherapy. [9] The drug treatment, incorporates Benzodiazepines, Selective serotonin reuptake inhibitors (SSRI), anxiolytics (non-addictive), β-blockers, and Tricyclic antidepressants (TCAs), its current use as anxiolytics. [10] That benzodiazepines currently do not recommend as first-line treatments due to their potential side effects. [11] and most of them are not curative; thus, exploring some new anxiolytics is required.

Drug repurposing is a different approach to recognizing the new indications for already approved drugs. [12] It is also represented as re-profiling, repositioning, re-tasking, and rescuing drugs. [13] In this process, hidden therapeutic functions of the drugs are uncovered using different approaches. [14]

Pitavastatin was launched in Japan in 2003 and is used clinically as a hypolipidemic drug in most countries. [15] Statins are well known for their LDL-lowering effect. besides this effect, it also helps to reduce pro-inflammatory markers like interleukin-1 (IL-1), IL-6, TNF-α, cyclooxygenase 2 (COX)-2, nitric oxide, reactive oxygen species (ROS), CRP, and prostaglandin E2 (PGE2). [16] There is not much data on inflammation linked with anxiety, but the level of circulatory markers was found to be elevated in patients with anxiety disorders. [17] By altering serum cholesterol levels, statins modulate serotonin transporter (SERT) functions. It improves mood functions by amplifying the N-methyl D-aspartate receptor (NMDA). [18]

Gemfibrozil is an FDA-approved drug that causes a decrease in serum cholesterol and triglyceride levels and increases high-density lipoprotein. [19] PPAR-α, through cyclic-AMP response element-binding protein (CREB), helps in synaptic plasticity via different genes. [20] such as the formulation of N-methyl-d-aspartate (NMDA) receptor subunit NR2A and NR2B genes. [21] Few studies suggest that through this pathway, PPAR-α might have a role in anxiety. The model of elevated plus maze is adopted for discovering anxiolytic behavior and it is reported in Fogg 1996; Rodgers and Johnson 1997. [22] In this pre-clinical study, we are trying to find out the anxiolytic aspects of pitavastatin and gemfibrozil in psychopharmacology, primarily used as hypolipidemic agents.

2-Material and Methods

2.1 Animal: Our animal study was done in the Pharmacology & Therapeutics department, King George's medical university, situated in Lucknow. Ethical approval was procured via Institutional Animal Ethics Committee (IAEC). (Ethical approval number- 150/IAEC/2021) 24 adult healthy male balb/c mice weighing 17-24 gm were utilized in our experiment. Balb/c mice were purchased through the Indian Institute of Toxicology Research [IITR] Lucknow. IITR is one of the affiliated centers of the "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA). They were protected in appropriate-sized cages in an Institutional animal house maintaining a specific temperature-controlled environment [26±2◦C], humidity (61% ± 10%) with 12 hours dark / 12 hours light pattern. Mice were provided a regular

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pellet diet with water according to their requirements. The regular pellet diet was procured through Bharat Science Solution Company, Unnao, Uttar Pradesh. All mice were permitted to familiarize themselves with the new territory for a couple of weeks in the animal house of Pharmacology department. Present validated models of rodents were selected to assess the anxiolytic properties of pitavastatin and gemfibrozil. Mice will be randomly divided into 4 different groups, each group containing 6 mice.

2.2 Drug treatment:

Tests drugs were solubilized in 0.5% carboxymethylcellulose (CMC) and dissolved in normal saline then given orally (p.o.) by a feeding gavage. The drug doses were selected from previous studies on the anxiolytic effect. Pitavastatin and gemfibrozil were administered to individual mice in groups 2, and group 3 subsequently. None of the mice was dead because of the treatment till the end of the experimental period.

2.3 Drugs: Pitavastatin, gemfibrozil, and diazepam were purchased from Gyan Scientific Traders Pvt. Ltd. Authorized company.

2.4 Vehicle: Pitavastatin and gemfibrozil was dissolved in 0.5%w/v CMC (carboxy-methylcellulose) and provided orally in mice (vehicle used normal saline). Diazepam was dissolved in normal saline and given i.p.

2.5 Behavioral model:

The elevated plus maze model is broadly adapted for evaluating anxiolytic properties. Maze generally uses for mice studies are comparably smaller in size than mazes for rats. Our study maze consisted of a couple of open arms (16.5 cm × 5cm) and two closed arms (16.5 cm× 5cm× 13cm) outstretched from a mid-square (5cm×5cm) and the maze was heighted up to 25cm from the base. On the first day, each mouse was put down at the center of the maze, facing toward the open arm. [23] The experiment was conducted in silent, soft lightroom. Mice were given a single dose of control, tests, and standard compounds. Mice were placed in a maze after 60min of drug administration. A definite five-minute experiment was performed for each candidate. The elevated maze was thoroughly mopped via 75% ethanol in between mouse testing periods. [24] All four paws onto and two paws outside of an arm represented an arm's arrival and departure.

Table 1: Animal grouping

ACTIVITY TO BE TESTED	GROUPS	TREATMENT
ANTI-ANXIETY EFFECT	GROUP 1	NORMAL SALINE
	GROUP 2	INJ. PITAVASTATIN 30 mg/kg BW
	GROUP 3	TAB GEMFIBROZIL 60 mg/kg BW
	GROUP 4	INJ DIAZEPAM 1 mg/kg BW

Assessment

(1) time duration spent at the open arms respective to the entire time duration consumed in the open and closed arms, calculated as a percentage (100 × times spent at open arm/total time in the plus-maze) and

(2) the total counting of entries in the open arm respective to all entries in open and closed arms, calculated as a percentage (100 × open/total entries).

2.6. Statistical analysis

The calculated data were demonstrated as Mean ± SD from 6 balb/c mice. Outcomes were applied to statistical analysis with help of one-way ANOVA supervened by post hoc Tukey's test to determine the significant variations if any among the groups. Calculated values defined as significant if the P value <0.05. Paired t-test used for calculating intragroup comparison. Data were analyzed by using excel and SPSS software.

Results:

3.1 Assessment of anti-anxiety activity (percentage open arm entries):

The antianxiety activity was estimated by the mean ratio number of entries in the open arm of the plus maze. [group 1: control, group 2: pitavastatin (test 1), group 3: gemfibrozil (test 2), group 4: standard (diazepam)]. Intergroup comparison was done by ANOVA and has been summarized in table 2 and graphically in figure 1.

Table 2: Intergroup comparison of the ratio of entries (open arm) in the elevated plus maze

Group	DAY 1		DAY 5	
	Mean	SD	Mean	SD
Group 1 (NS)	39.07	7.04	39.81	7.38
Group 2 (test 1)	40.73	8.36	53.33	10.03
Group 3 (test 2)	39.81	7.38	55.36	10.43
Group 4 (Standard)	41.39	6.70	58.14	10.92
ANOVA	F= 0.11; p = 0.95		F= 4.1; p = 0.019*	

*Statistically significant

N=24, n=6 in every group. Values are revealed as Mean ± SD posthoc Tukey's test was applied to find the significant variations after the application of one-way ANOVA

F = 0.11; P =0.95 (1^{st} day),

F = 4.1; P = 0.01* (5^{th} day),

Table 3: Between-group comparison of the percentage of open arm entries (Tukey HSD test)

Group	**DAY 1**			**DAY 5**		
	Mean Diff.	**SE**	**'p'**	**Mean Diff.**	**SE**	**'p'**
1 vs 2	-1.66	3.02	0.97	-13.51	3.99	0.11

1 vs 3	-0.74	3.02	0.99	-15.55	3.99	0.055
1 vs 4	-2.32	3.02	0.94	-18.33	3.99	0.01*
2 vs 3	0.92	3.02	0.99	-2.03	3.99	0.98
2 vs 4	-0.66	3.02	0.99	-4.81	3.99	0.83
3 vs 4	-1.58	3.02	0.98	-2.77	3.99	0.96

*Statistically significant

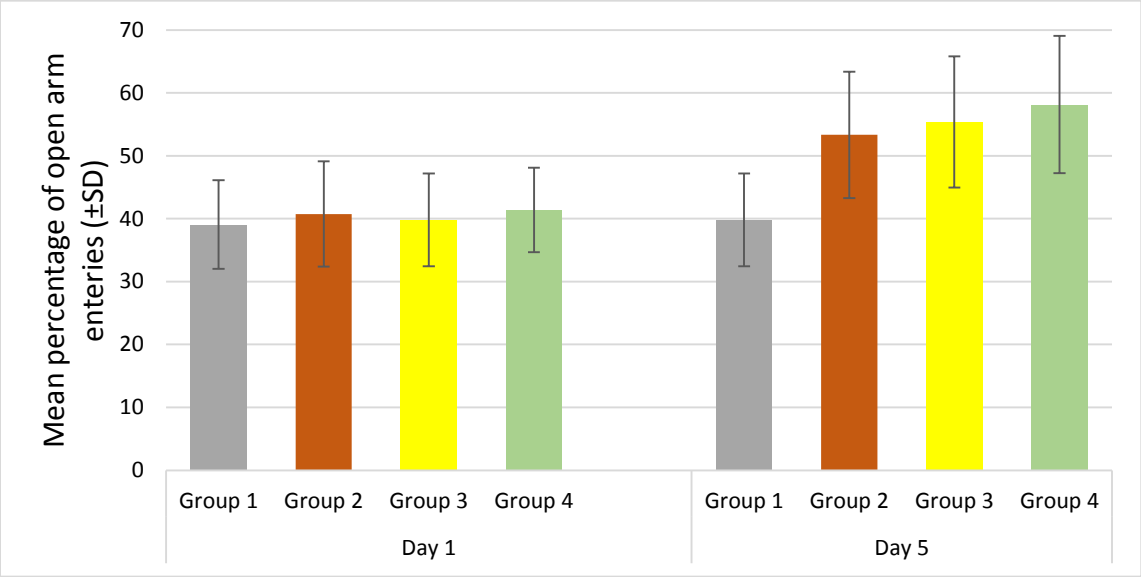

Figure 1: Percentage mean entries (open arms)

Intergroup comparison of entries (open arm):

On Day 1, The difference in the percentage count of entries at open arms on elevated plus maze by all above four groups was found comparable.

On day 5, a significant variation in the percentage count of entries (open arms) in the elevated plus maze was observed in the above four groups. On intergroup comparison, group 4 (58.14 ± 10.92) had a significantly higher mean ratio of open arm entries as compared to group 3 (55.36 ± 10.43), followed by group 2 (53.33 ± 10.03), and group 1 (39.81 ± 7.38). The number of percentage entries in group1 was significantly decreased in contrast to group 4.

Table 4: Intragroup change in Baseline (Day 1) %age number of open arm entries (paired 't-test) as compared to (Day 5)

Group	**Mean change**	**% BL change**	**t'**	**p'**
Group 1	-0.74	-1.85	0.14	0.88

Group 2	-12.59	-23.61	2.92	0.03*
Group 3	-15.55	-28.09	2.79	0.03*
Group 4	-16.74	-28.79	3.11	0.02*

*Statistically significant

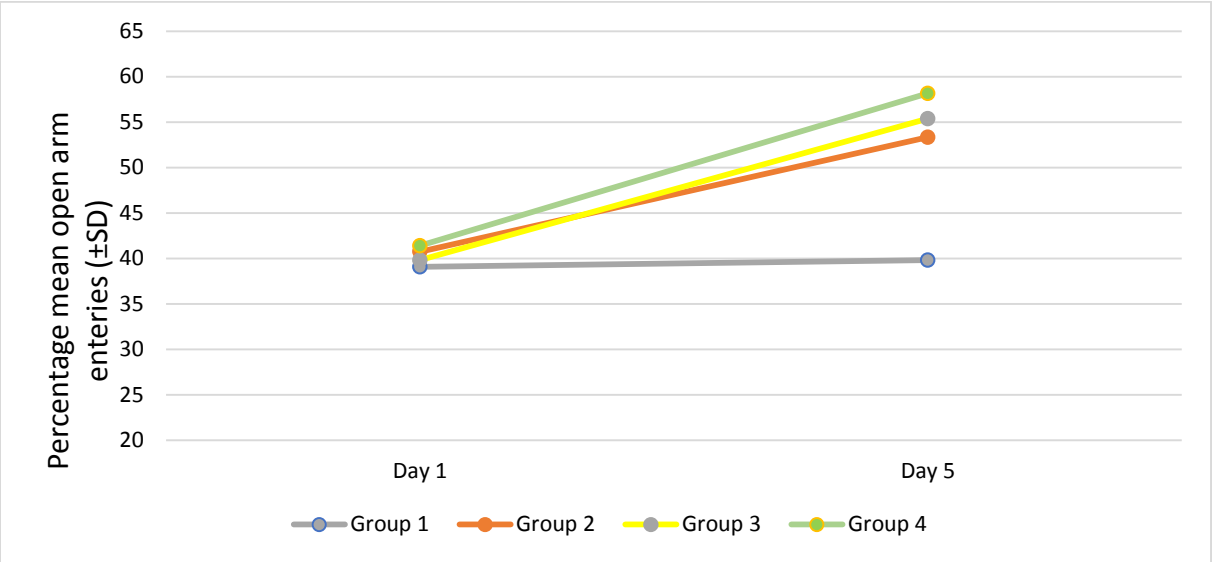

Figure 2: Comparison of the percentage of mean variations in the entries of open arms

Intragroup comparison: An increment in the percentage number of open arm arrivals on plus maze (elevated) was noted down from Day 1 to Day 5 in all groups. Changes in the number of visits in all groups were found significant except in group 9. The maximum change was detected in group 4 (28.79%) followed by group 3 (28.09%) and group 2 (23.61%) while the least change was observed in group 1 (1.85%).

3.2 Assessment of anti-anxiety activity (percentage time duration consumed in open arms): The antianxiety activity was estimated by the percentage of total time duration consumed in the open arm in the elevated plus maze. [group 1: control, group 2: pitavastatin (test drug 1), group3: gemfibrozil (test drug 2), group 4: standard (diazepam)]. Intergroup comparison was done by ANOVA and has been summarized in table 5 And graphically in figure 3.

Table 5: Intergroup comparison of %age of duration spent on the elevated plus maze (open arm)

Group	DAY 1		DAY 5	
	Mean	SD	Mean	SD
Group 1 (NS)	37.44	4.24	38.71	5.53
Group 2 (test 1)	40.27	6.76	50.10	6.57

Group 3 (test 2)	39.88	6.99	48.60	3.96
Group 4 (standard)	43.82	7.15	54.49	4.52
ANOVA	F= 1.01; p = 0.40		F= 9.67; p = 0.0003*	

*Statistically significant

N=24, n=6 in each group. Data were analyzed as Mean ± SD posthoc Tukey's test was applied to find the significant variations after the application of one-way ANOVA

F = 1.01; P =0.40 (1st day),

F = 9.67; P = <0.001* (5th day),

Table 6: Between-group comparison of percentage time spent in open arm (Tukey HSD test)

Group	**DAY 1**			**DAY 5**		
	Mean Diff	**SE**	**'p'**	**Mean Diff**	**SE**	**'p'**
1 vs 2	-2.83	2.61	0.86	-11.38	2.14	0.006*
1 vs 3	-2.44	2.61	0.91	-9.88	2.14	0.01*
1 vs 4	-6.38	2.61	0.35	-15.77	2.14	0.0002*
2 vs 3	0.39	2.61	0.99	1.5	2.14	0.95
2 vs 4	-3.55	2.61	0.77	-4.39	2.14	0.48
3 vs 4	-3.94	2.61	0.71	-5.89	2.14	0.24

*****Statistically significant

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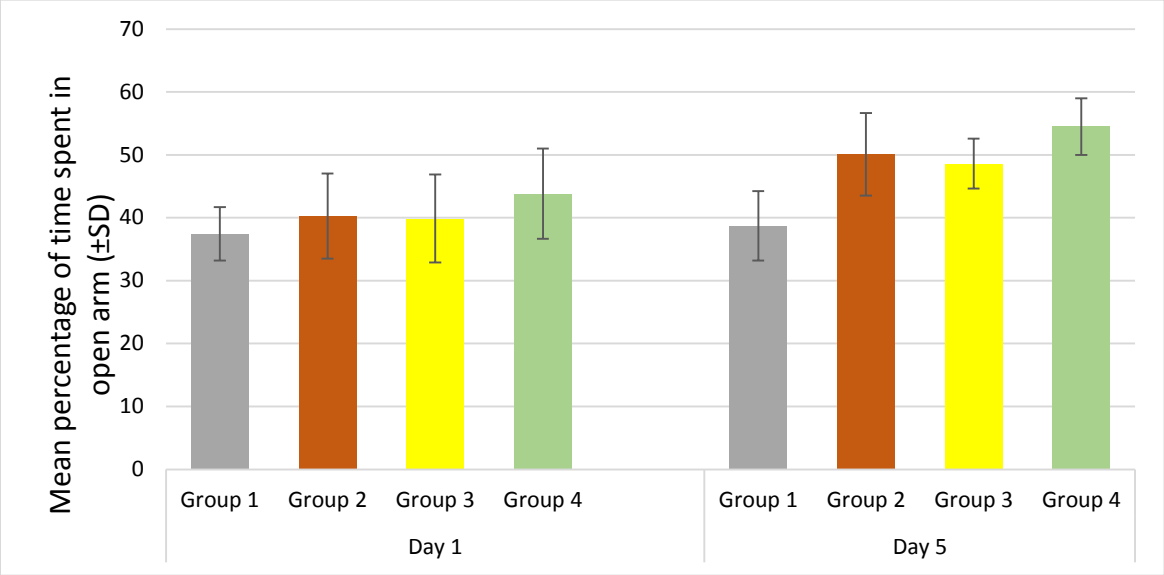

Figure 3: Percentage mean of time spent in open arm

Intergroup comparison of percentage time duration spent in the open arm:

On Day 1, The difference in the percentage duration consumed in open arm on elevated plus maze by all above four groups is found comparable. However, Group 12 shows an increment in the ratio of time spent (43.82 ± 7.15)

On day 5, a significant difference in the percentage of time consumed in the open arm on the elevated plus maze was observed in the above four groups. On intergroup comparison, Group 4 (54.49 ± 4.52) had a significantly higher count of open arm entries as compared to Group 2 (50.10 ± 6.57), Group 3 (48.60 ± 3.96), Group 1 (38.71 ± 5.53). The number of percentage time spent in the open arm of Group 1 was significantly lower than all the other three Groups.

Table 7: Intragroup change in Baseline (Day 1) percentage time consumed in open arm (paired ‘t-test) as compared to (Day 5)

Group	Mean change	% BL change	t'	p'
Group 1	-1.27	-3.30	0.37	0.72
Group 2	-9.83	-19.61	3.22	0.02*
Group 3	-8.72	-17.94	2.47	0.056
Group 4	-10.66	-19.57	6.99	0.0009*

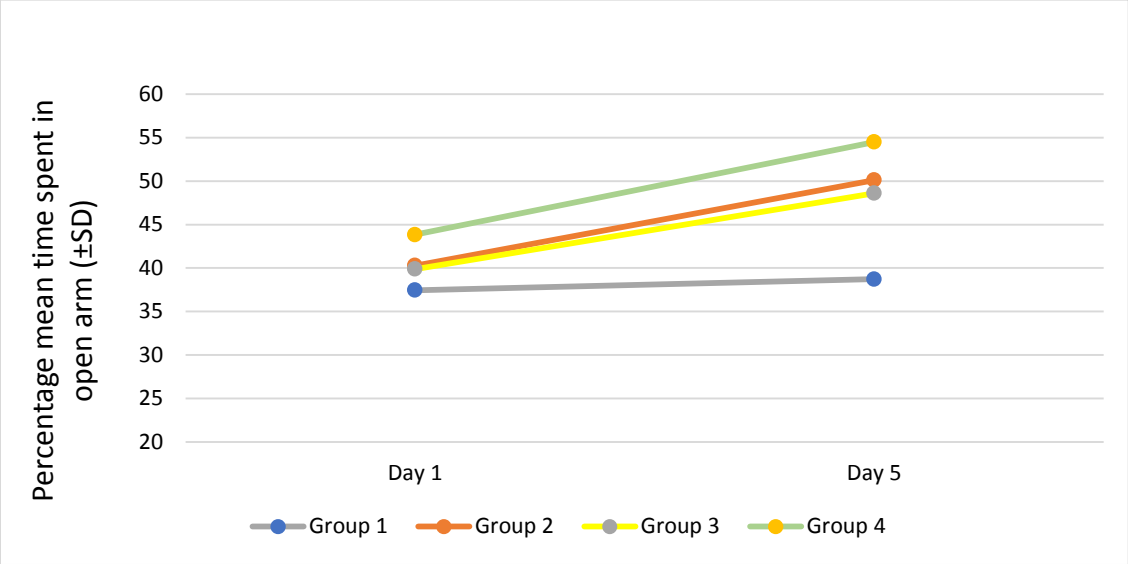

Figure 4: Comparison of the percentage of mean variations in time spent in open arm

Intragroup comparison: An increment of the mean ratio number of entries on open arms at elevated plus maze was observed on Day 1 to Day 5 in all groups, however, group 1 shows a minor change. Changes in percentage time spent were found to be significant in group 2 and group 4. The maximum change was detected in group 2 (19.61%) next by group 4 (19.57%) and group 3 (17.94%) while the least change was observed in group 1 (3.30%).

Conclusion:

Pitavastatin and gemfibrozil, are known for their hypolipidemic actions. There are research-based shreds of evidence that beyond their lipid-lowering effect, statins have several additional beneficial properties. [25] Elevated plus maze is a validated test to assess the anxiolytic power of pharmacological compounds and is widely used for behavioral assay. [26] The normal tendency of rodents to avoid entry in open arms of the elevated plus maze, more entries in the open arm typically show an antianxiety effect. The presence of free spaces and height in the surroundings is counted as the antianxiety effect of the pharmacological agent. [27] **In the present study** non, habituated mice were put down on the elevated plus maze. The total count of visits and the total time duration consumed in open and closed arms were observed for 5 minutes. Finally, the calculation was done by assessing the mean ratio of the number of visits in the open arm and the mean ratio of time duration consumed in the open arm. On **Day 1**, Concerning, the percentage number of entries in the open arm was found to be comparable. After 5 days of dosing with test drugs, the effect of the standard drug (diazepam) was found significant in intergroup contrast with the saline group. A significant difference in the percentage number of arrivals in the open arm of the elevated plus maze was observed in the above four groups. The percentage change in baseline was detected maximum in group 4 (28.79%) followed by group 3 (28.09%) and group 2 (23.61%) while the least change was observed in group 1 (1.85%).

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Concerning percentage of **duration consumed in the open arm** on **Day 1**, was found to be comparable. After 5 days of treatment with drugs a significant difference in the percentage of time spent in the open arm of the plus maze was observed in the above four groups. On intergroup comparison, a significant difference was found between control vs pitavastatin, control vs gemfibrozil, and control vs diazepam. The percentage change in baseline was evaluated maximum in group 2 (19.61%) next by group 4 (19.57%) and group 3 (17.94%) while the least change was observed in group 1 (3.30%).

The antianxiety effect of pitavastatin is probably due to the mechanism that statins have an inhibitory action on pro-inflammatory markers, such as TNF-α and IL-6. Recent studies show that pro-inflammatory markers have a role in the molecular process of anxiety. [28] The antianxiety effect of gemfibrozil is probably due to its role in basic peroxisome proliferator-activated receptor-alpha (PPAR-α). PPAR-α is known for its anti-inflammatory and neuroprotective role. [29] Synaptic plasticity of neurons will help in the normalization of anxiolytic behavior. That's why maybe pitavastatin and gemfibrozil have shown anti-anxiety effects.

Conclusion:

Both pitavastatin at dose 30 mg/kg and gemfibrozil 60 mg/kg BW represent an increment in the mean ratio of entries (open arm) and mean percentage of duration consumed in the open arm. Hence, it can be concluded that pitavastatin and gemfibrozil possess anti-anxiety effects but the effect was less than diazepam. Single research does not have the power to conclude the final result. Furthermore, research is needed to find out the exact role of these hypolipidemic drugs on anxiety behaviour.

Conflicts of interest

There are no conflicts of interest.

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