Bull. Pharm. Sci., Assiut University, Vol. 47, Issue 2, 2024, pp. 1203-1214.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/

BULL. PHARM. SCI. Assiut Univ.

NEURONAL GROWTH REGULATOR-1 (*NEGR1)* AND BRAIN-DERIVED NEUROTROPHIC FACTOR (*BDNF)*: NOVEL TARGETS OF COMBINED FENOFIBRATE AND PIOGLITAZONE TREATMENT OF OBESITY INDUCED DEPRESSION IN RATS

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Purpose: *Depression and obesity are closely related diseases sharing common biological mechanisms. PPAR- α and PPAR γ agonists have been hypothesized to improve memory and reduce neuroinflammation. Their exact role in treatment of obesity induced depression has been less understood. The study aims to investigate the potential molecular anti-inflammatory and anti-depressive effects of fenofibrate and/or pioglitazone on obese rats by targeting NEGR1 and BDNF expression.* ***Experimental****: Fifty male Swiss rats were equally divided into 5 groups: Group (i): Control group fed with standard rodent chow. Group(ii) High fat diet (HFD) group.Group (iii) Fenofibrate treated group. Group (iv) Pioglitazone treated group. Group (v) Fenofibrate+ pioglitazone treated group. Fenofibrate (18 mg/kg/day) and pioglitazone (3 mg/kg/day) were administered once daily for 4 weeks for rats fed on HFD for 8 weeks. Their effects on obesity-induced depression were evaluated through performing behavioral assessment such as forced swim test (FST) and tail suspension test (TST) , biochemical assessment(lipid profile) and molecular assessment (IL-6, TNF- α , neuronal growth regulator-1(NEGR1) and brain-derived neurotrophic factor (BDNF) expression using RT-PCR.* ***Results:*** *Obese rats manifested severe depressive-like behavior compared to controls, The combined fenofibrate and pioglitazone treatment significantly reduced the body weight, TG , LDL-C levels, downregulated the inflammatory markers and upregulated NEGR1 and BDNF expression compared to pioglitazone treated group.* ***Conclusion:*** *combined fenofibrate and pioglitazone treatment improve depression and obesity in obese rats via the upregulation of brain NEGR1 and BDNF expression holding tight interactions between brain, adipose tissue, and immune system.*

Keywords: *Fenofibrate , Pioglitazone,NEGR1, BDNF, Depression*

INTRODUCTION

Obesity and depression are two common pathologies having serious influence on the patients' health and on the socioeconomic attributes[1].

Numerous epidemiological and clinical studies related the link between mood disorders and obesity to obesity severity, gender, socioeconomic status, genetic susceptibility and environmental factors [2].

However, the relationship is likely multifactorial and complex, focusing on the common biologic mechanisms that can clarify the depression-obesity association at different levels, ranging from genetics to peripheral endocrinology mechanisms, immuno-inflammatory and metabolic mechanisms [3].

Various researches have found significant increases in plasma or serum levels of interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, and tumor necrosis factor- alpha(TNF- α)

Received : 18/4/2024 & Accepted : 20/6/2024

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[4].According to the "cytokine hypothesis of depression," these cytokines play a causal role in the progression of depression [5].

Moreover, in mouse models, emotional changes associated with inflammation-induced activation of the hippocampal indoleamine 2,3-dioxygenase enzyme (IDO) have been linked to lower hippocampal expression of the brain-derived neurotrophic factor (BDNF). In the hippocampus and other brain regions involved in mood regulation and learning, BDNF plays an important role in synaptic plasticity and neuronal survival [6].

Furthermore, in human and animal models, decreases in expression and mutations in the BDNF coding gene have been related to obesity [7].In addition, it was reduced in the hippocampus of stressed mice [8].

Neuronal growth regulator 1 (NEGR1) was identified as a locus associated with human obesity in a genome-wide association study (raft component from rat brain) and a neuronal influencer protein that control body weight and energy balance [9–12]**.** It is highly expressed in the cerebral cortex and hippocampus of the rat brain[13]. NEGR1 expression regulates hippocampal neuron synapse formation and promotes neurite outgrowth in mature cortical neurons[14].

Fenofibrate is used to treat adults who have primary hypercholesterolaemia, or hypertriglyceridaemia[15,16].

Pioglitazone is an anti-diabetic medication that acts as a peroxisome proliferator-activated receptors (PPAR)-γ agonist. PPAR-γ agonists have been shown to improve a variety of neurological disorders, including Alzheimer's disease and depression [17,18] by inhibiting the expression of inflammatory genes and improving insulin sensitivity, learning and memory [19].

Regarding the link between pioglitazone and BDNF, it is known that chronic administration of pioglitazone reverses memory impairment, BDNF disruption, and oxidative damage caused by beta-amyloid in an animal model of Alzheimer's disease [20–23]**.**

However, the exact mechanism that clarifies PPAR receptor agonists anti-depressant role isn't yet clearly identified. Therefore, this study was conducted to investigate the possible molecular anti-inflammatory and anti-depressive effects of fenofibrate and/ or pioglitazone on obesity - induced depression in rats by targeting BDNF and NEGR1 expression.

EXPERIMENTAL

Experimental animal procedures

Fifty male Swiss rats (180-200 g) were purchased from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt). Animals were allowed free access to food and water *ad libitum* and left for acclimatization for 7 days before the start of experiments.

Drugs and chemicals

Fenofibrate and Pioglitazone were prepared by dissolving in distilled water and given orally once daily by gastric tube in a dose of 50 mg/kg/day[24,25] and 20mg /kg /day respectively for 4 weeks [26,27].

Study Protocol

Rats were fed a HFD prepared by mixing 2% cholesterol, 0.3% bile salts and 10% lard with the basal diet[28] for eight weeks .

Rats (n = 50) were randomly allocated into 5 groups (n: 10/group) and assigned as follow: Group (i): Control group: rats fed with standard rodent chow. Group (ii) HFD control group: rats fed with HFD for eight consecutive weeks then, received distilled water orally daily by gastric gavage for 4 weeks. Group (iii) Fenofibrate treated group: rats fed with HFD for eight consecutive weeks then received fenofibrate for 4 weeks .Group (iv) Pioglitazone treated group: rats fed with HFD for eight consecutive weeks then received pioglitazone for 4 weeks . Group (v) Fenofibrate+ pioglitazone treated group: rats fed with HFD for eight consecutive weeks then received fenofibrate and pioglitazone for 4 weeks.

Behavioral studies, biochemical Assessment and molecular assessments

- Tail Suspension Test (TST)
  In brief, Rats were suspended approximately 28 ± 2 cm off the floor by hooking their tail (2 cm from the tip of the tail). During the experiment, rats

immobility time was automatically recorded for 6 minutes. [29,30].

- Forced Swimming Test (FST)
  Rats were forced to swim for 6 minutes in a transparent cylindrical container (40 cm high and 20 cm diameter) filled with clean water (24 ℃, 20 cm depth). The duration of the immobility state was determined. [29].
- Blood was collected from anaesthetized rats (80 mg/kg ketamine HCL, i.p.) at the end of the study. Blood samples were kept on ice until centrifugation (1500 × g for 7 minutes /room temperature) and sera were collected and stored at -80 ℃ for estimation of triglycerides, HDL,VLDL and LDL-C by sinus retro-orbital route for collection of plasma in a tube containing 10 ml of heparin solution and centrifuged at 10,000 rpm for 15 minutes using GPO-PAP-enzymatic colorimetric method. Commercially available kits were used to calculate triglycerides, HDL, VLDL and LDL-C
- After anaesthesia, the animals were perfused with phosphate buffered solution (PBS) at a pH of 7.4 through a cannula inserted in the left ventricle, followed by 4% paraformaldehyde. After perfusion, the brains were immediately removed and fixed in 4% paraformaldehyde in PBS at 4 ℃ for 12 hours before being embedded in optimum cutting temperature compound (OCT) and 20 mm tissue sections were air-dried at 20 ℃ before being moved to 80 ℃ for biochemical and molecular studies.
- RNA extraction and Real time PCR

At the end of each experiment, the hippocampus was isolated using a microdissection procedure. Total RNA was isolated from homogenized hippocampus tissue with a total RNA purification kit provided by Jena Bioscience (Munich, Germany) and stored at −80 ℃. RNA was converted into its complementary DNA by cDNA archive kit (Applied Biosystems, Foster City, California, USA). qPCR was performed by using GoTaq PCR master mix (Promega Co., Madison, USA). A protocol that included an initial denaturation step at 95 ℃ for 10 minutes, followed by 40 cycles of denaturing at 95 ℃ for 15 seconds, annealing and extension at 60 ℃ for 1 minute then 60 ℃ for 30 seconds was performed on a Step One Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The following oligonucleotide primers were used: 5'-CCTGGACGCAGTGGACTGAT -3' (sense), 5'- TGCTCCTGTGTCACGTTGGT -3' (antisense) for NEGR1*(Gene bank accession number is XM_032897116.1)*, 5′-GCGGCAGATAAAAAGACTGC -3′(sense), 5′- CAGTTGGCCTTTTGATACCG -3′ (antisense) for *BDNF (Gene bank accession number is XM_051183802.1),* 5'-ACTTCACAAGTCGGAGGCTT -3' (sense), 5'- AGTGCATCATCGCTGTTCAT -3' (antisense) for *IL-6 (Gene bank accession number is XM_032905335.1)*,5'-GCAGGACTTCTTCAGCGGACATG -3'(sense), 5'-GTTAGGTTCAGCTCGCCTCTTCAC -3' (antisense) for *TNF-α (Gene bank accession number is AJ012603.1)*, and 5′-TCCGTCGCCGGTCCACACCC -3′ (sense) and 5′- TCACCAACTGGGACGATATG -3′ (antisense) for *β-actin (Gene bank accession number is NM_031144.3)* as an internal control for normalization of NEGR1, BDNF, IL-6 and TNF-α target genes according to $2^{-\Delta\Delta ct}$ method.

Statistics

All the data are expressed as mean ± S.E and analyzed using statistical package for social sciences (SPSS) program version 17. All the comparisons among groups were carried out using one - way analysis of variance (ANOVA) followed by post-hoc multiple comparison; Bonferroni test, to test the significance difference among group means. Data were considered statistically significant with a P value < 0.05.

RESULTS AND DISCUSSION

Results

Effect of Fenofibrate and /or Pioglitazone on body weight in HFD- induced depression in rats

Table 1 shows that by the end of the experiment, high-fat fed rats showed

significant increase in body weight compared to control group. A statistically significant decrease in rat body weight was showed in fenofibrate treated group either alone or combined with pioglitazone compared with high-fat fed rats group.

Effect of Fenofibrate and /or Pioglitazone on serum lipid profile in HFD- induced depression in rats

Table 2 shows that high-fat fed rats exhibited hyperlipidemia, as is evident by the elevation in serum TG , LDL-C and VLDL levels and decline in HDL level compared to the normal control group ($p < 0.05$, **Table 2**). Treatment with fenofibrate and /or pioglitazone reduced the elevated TG , LDL-C and VLDL levels but enhanced HDL in comparison with HFD group ($p < 0.05$). Notably, fenofibrate treated group either alone or combined with pioglitazone exhibited a significant improvement in the severity of elevated TG and LDL-C levels compared to the pioglitazone treated group ($p < 0.05$, Table 2).

Effect of fenofibrate and /or pioglitazone on the brain relative expression of NEGR 1 and BDNF in HFD- induced depression in rats

Fig. 1 illustrates that both NEGR1**(Fig. 1a)** and BDNF expression **(Fig. 1b)** are downregulated ($P<0.05$) in HFD-fed rats in comparison to normal control rats Treatment with fenofibrate and /or pioglitazone revealed effectiveness in upregulating the expression of these markers ($p<0.05$) in comparison with HFD group. It was evident that treatment with combined fenofibrate and pioglitazone was more beneficial in attenuating these deleterious effects associated with HFD compared to pioglitazone treated group ($p<0.05$, **Fig. 1**).

Table 1 : Effect of Fenofibrate and /or Pioglitazone on body weight in HFD- induced depression in rats.

	Normal Control	HFD	Fenofibrate treated	*Pioglitazone treated*	*Fenofibrate+ pioglitazone treated*
Body weight (start) (g)	155.4 ± 0.5	147. 4±1.2	158. 6±0.56	151. 5±1.00	149.1±1.80
Body weight (end) (g)	207. 9 ±11.0	397.8±18.3¶	334.9±11.40*	340.1 ±9. 9*	319.50±14.07*

Rats were fed with a HFD for 8 weeks. HFD: high-fat diet. Results are expressed as mean ± S.E. and analyzed using one-way ANOVA followed by Bonferroni *post-hoc* test at $P<0.05$. ¶ Compared to normal control group, * Compared to HFD group, #Compared to pioglitazone treated group. *n = 10.*

Table 2 : Effect of Fenofibrate and /or Pioglitazone on serum lipid profile markers in HFD- induced depression in rats.

Groups	Triglycerides (mg/dl)	LDL-C (mg/dl)	HDL (mg/dl)	VLDL (mg/dl)
Normal Control	92.5 ± 6.1	22.0 ± 4.3	44.7 ± 3.0	18.5 ± 1.2
HFD	202.8 ± 29.1¶	93.5 ± 22.1¶	21.4 ± 3.5¶	41.4 ± 6.3¶
Fenofibrate treated	110.8 ± 7.2*#	23.5 ± 4.1*#	38.6 ± 2.1*	26.4 ± 2.5*
Fenofibrate+pioglitazone treated	138.3 ± 7.4*	52.5 ± 11.0*	42.2 ± 3.6*	24.7 ± 2.9*

Rats were fed with a HFD for 8 weeks. HFD: high-fat diet. Results are expressed as mean ± S.E. and analyzed using one-way ANOVA followed by Bonferroni post-hoc test at P<0.05. ¶ Compared to normal control group, * Compared to HFD group, #Compared to pioglitazone treated group. n = 10.

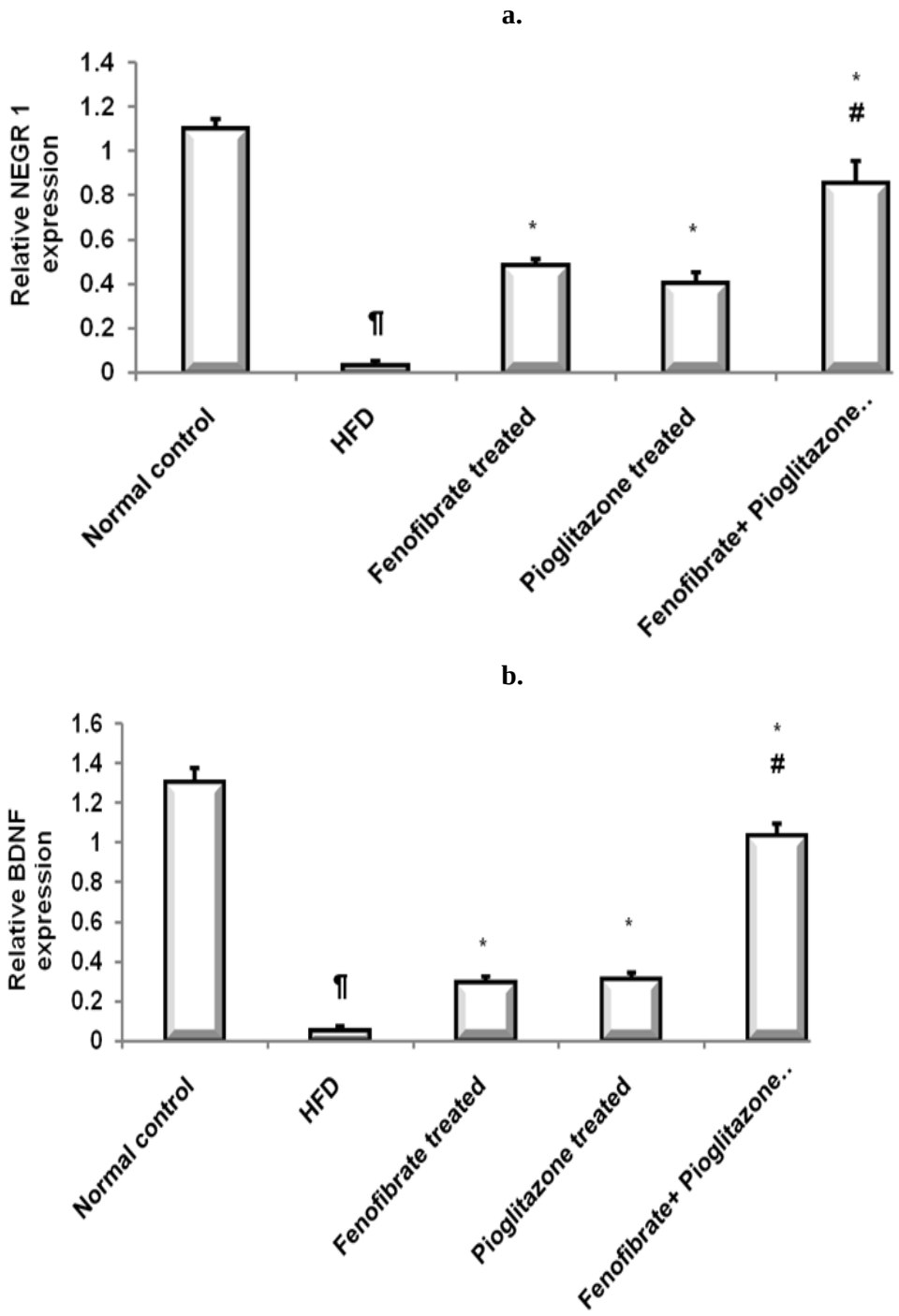


Fig. 1 Effect of Fenofibrate and /or Pioglitazone on brain relative expression of (A: NEGR 1, B: BDNF) in HFD-induced depression in rats. Values are mean ± S.E. (n= 10), analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test. ¶,*, # p< 0.05; ¶ Compared with normal control group, * Compared with HFD control group, # compared with pioglitazone treated group.

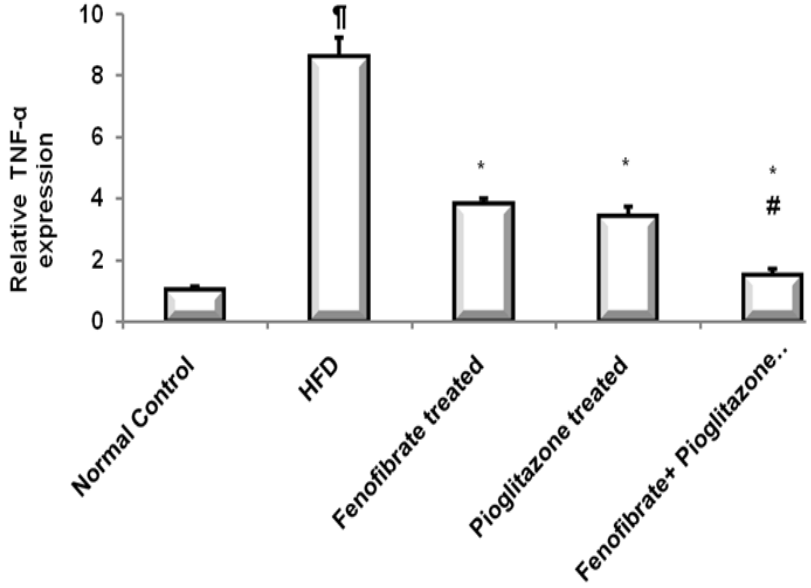
Effect of fenofibrate and /or pioglitazone on the brain relative expression of TNF-α and IL-6 in HFD- induced depression in rats

Fig. 2 shows that HFD was associated with increased brain mRNA expression of both TNF-α **(Fig. 2a)** and IL-6 **(Fig. 2b)** in comparison with normal control group ($p<0.05$, **Fig.2**). Treatment with fenofibrate and /or pioglitazone downregulated the expression of these inflammatory markers ($P<0.05$) compared to the HFD control group. Notably, the combined fenofibrate and pioglitazone treated group effectively downregulated the expression of both TNF-α and IL-6 compared to pioglitazone-treated group ($p < 0.05$, Figure 2).

Effect of fenofibrate and /or pioglitazone on the depressive behavior in HFD- induced depression in rats

In **Fig. 3**, It was obvious that, HFD was associated with depressive behavior as revealed by the increased (*P*< 0.05) immobility time when rats hanged passively in TST **(Fig.3a)** and the helpless behavior and passively floating in the FST **(Fig.3b)** compared to normal control group. Treatment with fenofibrate and /or pioglitazone reversed this depressed behavior in TST and reduced (P < 0.05) the immobility time of rats in FST versus HFD group **(Fig. 3).** Treatment with fenofibrate either alone or combined with pioglitazone improved the depressive behavior in FST compared to pioglitazone treated group (*P*< 0.05, **Fig. 3**).

a.



b.

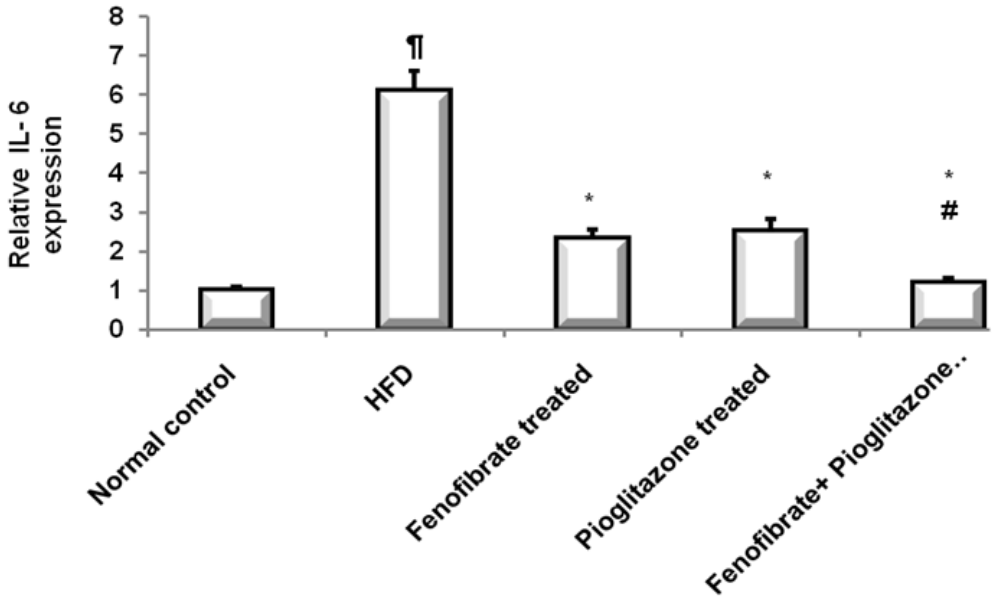


Fig. 2 Effect of Fenofibrate and /or Pioglitazone on brain relative expression of (A: TNF-α, B: IL-6) in HFD- induced depression in rats. Values are mean ± S.E. (n= 10), analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test. ¶,*, # p< 0.05; ¶ Compared with normal control group, * Compared with HFD control group, # compared with pioglitazone treated group.

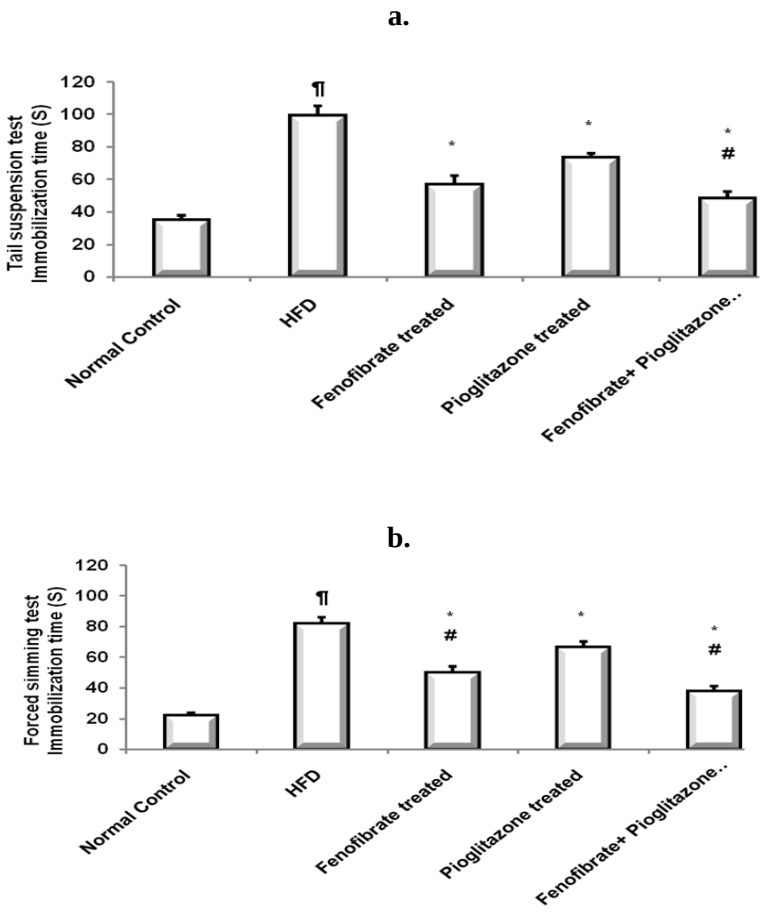


Fig. 3. Effect of Fenofibrate and /or Pioglitazone on the depressive behavior (A: tail suspension test,B: forced swimming test) in HFD- induced depression in rats. Values are mean ± S.E. (n= 10), analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test. ¶,*, # $p< 0.05$; ¶ Compared with normal control group, * Compared with HFD control group, # compared with pioglitazone treated group.

Discussion

Due to their rising prevalence globally, depression and obesity are currently significant public health concerns. Numerous clinical studies reveal a nuanced link that supports the idea that depression and obesity can interact in a reciprocal association[2]. It is frequently asserted that dietary fat intake is to blame for the rise in adiposity not only in humans but also in rats and mice which are considered as suitable models for investigating dietary obesity[31]. Intriguingly, obese mice exhibited symptoms of anxiety and depression as well since obesity evokes a neuro -inflammatory response in the brain [32].

In this study, we demonstrated that feeding rats HFD for 8 weeks led to a measurable rise in body weight linked to hyperlipidemia manifested by a robust elevation in serum TG , LDL-C and VLDL levels and decline in HDL level. In addition, it was evident that HFD was connected to depressive behavior as evident by the time that rats hung passively in the TST and passively floated in the FST. In addition, treatment with fenofibrate alone or combined with pioglitazone improved dyslipidemia and the depressive behavior in TST

Fenofibrate is one of a class of lipid-lowering medications known as fibrates that is used to treat individuals with hypertriglyceridemia and hypercholesterolemia through regulating fatty acid metabolism genes [33] . It contributes to decreasing the body weight by inducing fatty acid oxidation, inhibiting TAG synthesis and consequently lowering total cholesterol, triglycerides, and LDL cholesterol while increasing HDL cholesterol levels in obese mouse model[34].

According to Roy et al. (2013), PPAR-α was also abundantly expressed in the nuclei of hippocampus neurons and managed the expression of different proteins involved to plasticity by directly regulating the transcription factor known as cyclic adenosine monophosphate response element binding protein (CREB)[35].

Moreover, the classic explanation for pioglitazone's ability to treat type 2 DM and obesity is the PPARγ-mediated subcutaneous adipose tissue expansion, which lowers systemic lipid levels, reverses "lipotoxicity" in tissues and improves dyslipidemia, and insulin sensitivity[36]. In addition, pioglitazone decreased the visceral fat volume and its metabolic activity in patients with type 2 DM [37] and could increase the adiponectin transcription [38].

However, the exact mechanism that clarifies the anti-depressant role of fenofibrate and pioglitazone isn't yet clearly identified.

In this study, we investigated the effect of fenofibrate and /or pioglitazone on the brain relative expression of NEGR 1 and BDNF in HFD- induced depression in rats. Both NEGR1 and BDNF expression are downregulated in HFD-fed rats that were reversed by treatment with fenofibrate and /or pioglitazone

NEGR 1 is a GPI-anchored protein that belongs to the immunoglobulin LON family (IgLON) [39] and is considered as a main linker between depression and obesity by modifying the synaptic plasticity of the cortex, hippocampus, and hypothalamus regions of the brain that are essential for controlling mood and eating habits[3], signposting the crucial role played by CNS in the control of body weight and energy homeostasis, which overlap with those in charge of controlling mood.

Partly, NEGR 1 acts as synaptic adhesion molecule that controls the growth of mature cortical neurons' neurites as well the formation of hippocampal neurons' synapses [40].However, Multiple studies had demonstrated the downregulation of *NEGR1* expression in various psychiatric and behavioral disorders as depression, anxiety, autism and increased risk for seizures through alteration in adult neurogenesis and hippocampus synaptic transmission [11,39,41].

According to Kim et al., deficiency of NEGR1 may cause an excessive cholesterol deposition and encourage intracellular lipid storage [10]. In addition, Joo et al. concluded that NEGR1 deficiency induced lipid accumulation in mice liver, declaring that beside the central function of NEGR 1, it can act also peripherally since it exists in adipose and smooth muscle tissues, by affecting cholesterol trafficking [42]. So, targeting NEGR1 can greatly improve obesity associated with depression and help in reduction of body weight.

Similarly, in the present study, combined fenofibrate and pioglitazone was more beneficial in upregulating *BDNF* expression in HFD rats. BDNF holds the tight interactions between brain, adipose tissue, and immune system.

This neurotropic factor, considered also as an adipokine, affects glucose and insulin metabolism as Motamedi et al., denoted that *BDNF* mutations are associated with weight gain in both humans and mouse models associated with higher levels of serum cholesterol, leptin, glucose, fatty acids, as well as insulin resistance and obesity [43].

However, Adipocytokine production is dysregulated by oxidative stress in obese mice, which encourages inflammation and leads to low levels of BDNF [44].

Moreover, the relationships between depression and obesity also entail inflammatory and immune components. The proinflammatory cytokines (such IL-1, IL-6 and TNF-α), which are produced in response to the immunological stimuli, are the most significant molecules in the network of inflammatory mediators [2]. Consequently, this study declared a significant downregulation in the brain mRNA expression of both *TNF-α* and *IL-6* after the administration of the combined fenofibrate and pioglitazone in the treated group in comparison with HFD group.

Conclusions

This study highlighted the significant role of the combined fenofibrate and pioglitazone treatment in improving depression and obesity in obese rats via the upregulation of brain *NEGR1* and *BDNF* expression.

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Bull. Pharm. Sci., Assiut University, Vol. 47, Issue 2, 2024, pp. 1203-1214.

نشرة العلوم الصيدليـــــة
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BULL. PHARM. SCI. Assiut Univ.

منظم نمو الخلايا العصبية -١ (NEGR1) وعامل التغذية العصبية المشتق من الدماغ (BDNF) : أهداف جديدة للعلاج المشترك للفينوفايبرات وبيوجليتازون للاكتئاب الناجم عن السمنة في الفئران

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الغرض: الاكتئاب والسمنة من الأمراض المرتبطة ارتباطا وثيقا التي تشترك في الآليات البيولوجية المشتركة. تم افتراض منبهات PPAR- α و PPAR γ لتحسين الذاكرة وتقليل الالتهاب العصبي. كان دورهم الدقيق في علاج الاكتئاب الناجم عن السمنة أقل فهما.

تهدف الدراسة إلى التحقيق في التأثيرات الجزيئية المحتملة المضادة للالتهابات والاكتئاب للفينوفايبرات و / أو بيوجليتازون على الفئران البدينة من خلال استهداف تعبير منظم نمو الخلايا العصبية -١ NEGR1 وعامل التغذية العصبية المشتق من الدماغ .BDNF

التجربة : تم تقسيم خمسين من ذكور الفئران السويسرية بالتساوي إلى ٥ مجموعات: المجموعة (i): المجموعة الضابطة التي تم تغذيتها بسمك القوارض القياسي. المجموعة (ii) مجموعة النظام الغذائي عالي الدهون .HFD. المجموعة iii المجموعة المعالجة بالفينوفايبرات. المجموعة (الرابعة) مجموعة بيوجليتازون المعالجة. المجموعة (v فينوفايبرات + مجموعة بيوجليتازون المعالجة.

تم إعطاء فينوفايبرات (١٨ مجم / كجم / يوم) وبيوجليتازون (٣ مجم / كجم / يوم) مرة واحدة يوميا لمدة ٤ أسابيع للفئران التي تغذت على HFD لمدة ٨ أسابيع. تم تقييم آثارها على الاكتئاب الناجم عن السمنة من خلال إجراء تقييم سلوكي مثل اختبار السباحة القسري (FST) واختبار تعليق الذيل (TST)، والتقييم الكيميائي الحيوي (ملف الدهون) والتقييم الجزيئي (IL-6، TNF- α ، منظم نمو الخلايا العصبية -١ (NEGR1) وتعبير عامل التغذية العصبية المشتق من الدماغ (BDNF) باستخدام تفاعل سلسلة البلمرة .RT-PCR

النتائج: أظهرت الفئران البدينة سلوكا شديدا شبيها بالاكتئاب مقارنة بالضوابط ، وأدى العلاج المشترك بالفينوفايبرات والبيوجليتازون إلى تقليل وزن الجسم بشكل كبير ، ومستويات TG ، و LDL-C ، وتقليل علامات الالتهاب والتعبير NEGR1 و BDNF مقارنة بالمجموعة المعالجة ببيوجليتازوني.

الخلاصة: يعمل العلاج المشترك بالفينوفايبرات والبيوجليتازون على تحسين الاكتئاب والسمنة لدى الفئران البدينة عن طريق تنظيم تعبير الدماغ الذي يتم عن طريق NEGR1 و BDNF بواسطة عقد تفاعلات وثيقة بين الدماغ والأنسجة الدهنية والجهاز المناعي.