RESEARCH ARTICLE

Evaluation of Anti-aging Effects of Gemfibrozil on D-galactose induced Aging Mouse Model

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Received: 13th February, 2023; Revised: 20th March, 2023; Accepted: 08th August, 2023; Available Online: 25th September, 2023

ABSTRACT

Background: Aging is the sequential or progressive changes in the organisms that is associated with increasing susceptibility to disease and death. Advanced age is associated with increased incidence of a variety of chronic disease states that share oxidative stress and inflammation as causative role players. Accumulating evidence in published literature showed that the use of antioxidant and anti-inflammatory agents is an effective approach to alleviate or reverse aging-related changes with consequent reduction of the aging rate and related disease. Gemfibrozil, as a peroxisome proliferator-activated alpha receptor ligand (PPARα), is reported to have antioxidant and inflammatory activity.

Aim of the study: Evaluate the anti-aging effect of gemfibrozil on many parameters associated with the aging process on the D-galactose induced aging mice model.

Method: The current work was an experimental randomized controlled study in which 60 albino male mice weighing between 25 to 40 gm were randomly divided into 6 groups (10 mice each group). group1 apparently healthy group, receive normal saline orally. Group 2 (age induction group receive D-galactose 500 mg/kg) orally only for 6 weeks, group 3 (+D-galactose 500 mg/kg + vitc 100 mg/kg orally for 6 weeks) group 4 (D-galactose 500 mg/kg for 6 weeks then vitc 100 mg/kg for another 6 consecutive weeks group 5 (D-galactose 500 mg/kg + gemfibrozil 7.5 mg/kg concomitantly for 6 weeks) group 6 (D-galactose 500 mg/kg for 6 weeks then gemfibrozil 7.5 mg/kg for another 6 consecutive weeks. Then animals were sacrificed, liver and kidney were weighed for organ index measurement and tissue sections and heart homogenate were prepared for ELISA assay (measurement of TN alpha, IL1beta, SOD, GPX) and histopathological analysis of myocardial tissue.

Results: results showed a significant rise in liver and kidney indices in animals that received gemfibrozil orally administered (during and after induction) compared to aged group, with a dramatic decrease in inflammatory and oxidative stress mediator level and a marked reversal effect on myocardial hypertrophy induced by D-galactose.

Conclusion: Gemfibrozil oral administration alleviates aging-associated atrophic changes in liver and kidney, oxidative stress and inflammatory state in myocardial tissue and reverses aging-related myocardial hypertrophic changes.

Keywords: Aging, Gemfibrozil, D-galactose, Kidney.

International Journal of Drug Delivery Technology (2023); DOI: 10.25258/ijddt.13.3.40

How to cite this article: AL-Bairmani RJH, Kadhim HM. Evaluation of Anti-aging Effects of Gemfibrozil on D-galactose induced Aging Mouse Model. International Journal of Drug Delivery Technology. 2023;13(3):1011-1016.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Aging in humans is a complex process characterized by time-dependent functional decline, resulting in a decrease in the quality of life.[1] Age-related decline in function is a physiological phenomenon occurring in all organ systems. However, acceleration and early occurrence of this process are observed in cardiovascular pathologies.[2] It is mainly characterized by progressive pathological remodeling with stiffening.[3]

Among the more than 400 theories published to explain the aging process, one of the most widely accepted theories is the theory of free radicals, proposed by Harman,[4] and further developed by many researchers.[5]This theory indicates that aging is a consequence of the accumulation of damage by deleterious oxidation in biomolecules caused by the high reactivity of the free radicals produced in cells as a result of the necessary use of oxygen.[6] Although several authors reject this idea[7] the redox theory of aging appears to be accepted by many others.[8] Another relevant theory is that of "inflame-aging",[9] which points out the presence of mild inflammation in aging. More recently, the oxidation-inflammation theory of aging was proposed to combine the age-related increases of oxidation and

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inflammation as two intimately related processes,[10] In aged individuals, intense inflammatory activities characterized by the presence of cytokines, apoptotic cells, immune cell infiltration.[11,12] Several factors are responsible for inflammation, including dyslipidemia, hyperglycemia, elevated nuclear-factor kappa B (NFκB) activity, increased levels of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (ILs).[13-15] Studies showed that Excess reactive oxygen species (ROS) and superoxide generated by oxidative stress and low-grade inflammation accompanying aging recapitulate age-related cardiovascular dysfunction, that is, left ventricular hypertrophy, fibrosis, and diastolic dysfunction in the heart.[16]

There is great interest in drugs capable of modulating diverse aging pathways, delaying the onset and progression of aging.[17] Many researchers have investigated the role of many drugs and herbs in alleviating age-related changes utilizing their antioxidant anti-inflammatory activity.[18,19]

Gemfibrozil has been reported to control biological pathways independent of PPAR-α. Including anti-inflammatory and ant oxidative mechanisms of which l is believed to occur via PPAR-α-independent pathways.[20] It is well established that gemfibrozil inhibits the production and release of inflammatory mediators, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).[21]

METHOD

The current work was an experimental randomized controlled study. This work was conducted from January 2022 to December 2022 and carried out at the biotechnology research center, Al Nahrain University. All the experimental work was implemented following the protocol reviewed by the Institutional Review Board of the College of Medicine, Al-Nahrain University, after being approved by the scientific committee of the Department of Pharmacology at the College of Medicine, Al-Nahrain University**.**

Study Design

Male swiss albino mice between the ages of 3 and 4 months and average body weight of 20 to 30 gm, were randomly divided into four groups each consisting of 10 animals (40 mice in total). The animals were identified by marking different parts of the body. The mice were picked up from the National Center for Drug Control and Research, housed in a polypropylene cage under a temperature-controlled environment (22 ± 2°C), with an inverted light-dark cycle (12/12 hours) and acclimated for two weeks before the study (Animal Facility of the Al-Nahrain University–Biotechnology research center, Baghdad, Iraq). The animals were maintained on a standard pellet diet and free access to water *ad libitum*.

For Animal Allocation

The mice were randomly assigned into 4 groups (10 mice each)., group I acted as (control group). Apparently healthy control: given normal saline via gastric gavage once daily for 6 weeks, group II acted as (aging induction model) and received D-galactose (500 mg/kg) orally via gastric gavage once daily for 6 Weeks.[22] Group III (Vitamin C started after the day aging model was established): In detail 500 mg/kg of D-galactose given orally by gastric gavage once daily for six weeks and mice that attained success aging model allowed to complete the study, starting next day vitamin C 100 mg/kg was given orally by gastric gavage once daily and continued for another six weeks Group IV (Gemfibrozil started after the day aging model established): in details 500 mg/kg of D-galactose given orally by gastric gavage once daily for six weeks and mice that attain success aging model allowed to complete the study, starting next day gemfibrozil 7.5 mg/kg was given orally by gastric gavage once daily and continued for another six weeks, for more details see Figure 1.

D-Galactose was supplied as powder preparation by Sigma Aldrich®, USA, (CAS no. 59-23-4). Gemfibrozil was supplied as powder preparation by Hangzhou Hyper Chemicals Limited®, China (CAS no.25812-30-0), and vitamin C was supplied as powder preparation by Hangzhou Hyper Chemicals Limited®, China (CAS no.86404-04-8).

Induction of Aging in Experimental Animals

For this purpose, D-galactose is administered in a dose of 500 mg/kg orally via oral gavage feeding needles to 50 albino male mice once daily for 6 weeks to induce aging in mouse models. General mice conditions were observed to ensure that an accelerated aging animal model is achieved. Old mice developed signs like withered lackluster fur, wrinkled skin and a more rounded general appearance, and they seem less alert, less responsive with sluggish activity and appeared to be clustered together[23] as seen in Figure 2.

Sample Size and Randomization

To compute sample size, program G power was utilized,[24] based on Cohen's principles.[25] A table of random integers was used to set up the groupings randomly. To minimize misunderstanding and errors, the animals were placed in labeled cages and given tail tags[26]

Weight Measurement and Organ Index Calculation

Weight of animals in each group were measured at the end of the experiment and before terminating animals in order to calculate organ index after the end of the experiment, all mice were euthanized at the end of their agent's administration period, which is 12 weeks.

Following completion of therapy, all animals were starved for 12 hours and anesthetized After the by an overdose of diethyl ether inhalation end of the experimental period, dissection was done for the euthanized mice, heart, liver and kidneys were removed and weighed to determine the organ index based on the following equation described by Chen *et al.*

Organ index %= organ weight(g)/body weight (g)*100

Heart tissue was divided into 2 pieces one piece was used for histopathological analysis, first washed with "PBS, pH 7.4" Then and processed by the traditional processing procedure by the paraffin-embedded method The other piece

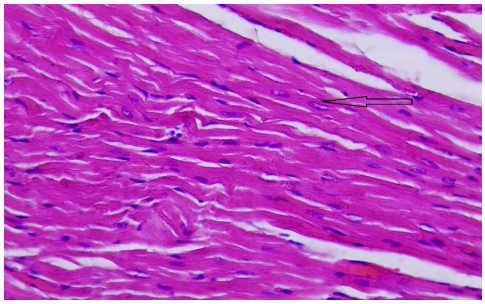

Figure 1: Longitudinal section of cardiac tissue showed normal cardiomyocytes with normal size nuclei (black arrow) hematoxylin and eosin stained under light microscope (BX5 microscope, X40) in mice received normal saline only (group I)

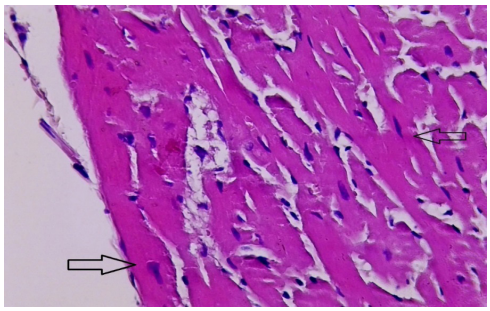

Figure 2: Longitudinal section of cardiac tissue showed hypertrophied cardiomyocytes with enlarged nuclei (black arrow). Hematoxylin and eosin stained under light microscope (BX5 microscope, X40)in mice received D-galactose only (group II)

of heart tissue was isolated and rinsed with cold phosphate buffer saline "PBS, pH 7.4", then the tissue was dried with filter paper and used for ELISA analysis by (ELISA reader, Diagnostic Automation/Cortez Diagnostics®, California, USA) and weighed by sensitive balance. For ELISA, each 50 mg of tissue was put in an Eppendorf tube (Eppendorf®, Hamburg, Germany) containing 0.45 mL of chilled PBS and then minced into small pieces. The tube containing the tissue was then put in an ice-containing beaker to keep it cold and then homogenized by the homogenizer machine (Electrical tissue homogenizer, Staruar®, England). The homogenate was centrifuged for 20 minutes at 4°C and 2000 rpm in a cold centrifuge (Thermos scientific®, USA). The supernatant was isolated using a micropipette (Bioevopeak®, China), and stored at -20°C until the day of analysis.

Laboratory Investigation

At the end of the experiment, after euthanization, the abdomen was dissected by a midline incision, and then the heart was isolated and rinsed with cold phosphate buffer saline ("PBS, pH 7.4") to get rid of residual blood and other debris, then the tissue was dried with filter paper and divided into two parts for ELISA and weighed by sensitive balance. For ELISA each 50 mg of tissue was put in an Eppendorf tube containing 0.45 mL of chilled PBS and then minced into small pieces. Eppendorf containing the tissue then was put in an ice-containing beaker to keep it cold and then homogenized by the homogenizer machine. The homogenate was centrifuged for 20 minutes at 4°C and 2000 rpm in a cold centrifuge. The supernatant was isolated using a micropipette and stored at -20°C until the day of analysis.

Biochemical Analysis

The resultant stored supernatant of the sampled mice homogenate heart tissues was then sent for biochemical analysis by double-sandwich ELISA technique for Tumor necrosis factor-alpha (TNF-α) by the kit (Mouse Tumor necrosis factor A, TNF-A ELISA KIT, product ID SL0547Mo, Sunlong biotech®, China), Interleukin-1Beta (IL-1β) by the kit (Mouse Interleukin1beta, IL-1beta ELISA Kit, , Sunlong biotech®, China), sodium oxide dismutase (SOD) Malondialdehyde (MDA) by the kit (Mouse SOD kit (MDA) by the kit (Mouse Malondehyde (MDA) ELISA Kit, product, Sunlong biotech®, China

Light Microscopy

Applied by counting the number of hypertrophic cells in 1mm area of tissue per 5 high power field (hpf) magnified by 400x. (modified from., Cree *et al.*[27] on WHO classification of tumor).[27] A digital microscope was used to randomly take five zones of a slide corner and the center at 40X magnification power.[28] Hypertrophic cells were identified by an increase in cardiac myocyte size in addition, cardiac myocytes are characterized by the presence of nuclei, which are also increased in size, irregular, and hyperchromatic.[29]

Statistical Analysis

Statistical Packages for Social Sciences (SPSS) software, version 25 was used to analyze data. Data were presented as mean ± SEM and one-way analysis of variance [ANOVA] with post-hoc test were used to compare the treatment effects between the groups, and a value of $p< 0.01$ was considered as statistically significant.[30]

Ethical Approval

The study was approved by the ethical committee of Al-Nahrain University, College of Medicine (Approval no.: 20210912, on 9th of December 2021).

RESULTS

Kidney index, there was a significant decrease in liver and kidney index mean of group II compared to group I at ($p \leq 0.01$). while groups III and group IV show high significant increase in organ index means in relation to group II at ($p \leq 0.01$), heart index means show a significant rise in group 2 and dramatically decreased in all other treatment groups at ($p \leq 0.01$), as shown in Table 1.

Results showed a highly significant increase in means of inflammatory mediators in heart tissue of group II at ($p \leq 0.01$) compared to the control group. On the other hand, the means of the group 3, 4 have a highly significant reduction in inflammatory mediators' level in comparison to group 2 at ($p \leq 0.01$), as shown in Table 2.

Results showed that mean levels (SOD) was significantly dropped in group 2 when compared to group 1 ($p \leq 0.01$) when compared to group 2. All other study groups show a high significant increase in mean of (SOD) levels at ($p \leq 0.01$).

Regarding MDA means levels, results showed that there is a significant rise in the level of MDA in group 2 when compared to group 1 at ($p \leq 0.01$), while all other treatment groups showed a very significant drop in the means of MDA level when compared to group 2 ($p \leq 0.01$).

Histopathological Findings

Hypertrophic changes in myocardial tissue of all study groups

Histopathological quantification of cardiomyocyte hypertrophy showed that the induced group showed the highest count of hypertrophic myocytes, characterized by larger cell volume and lager nucleus size in relation to control cells, the control healthy group showed a minimal number of hypertrophic cells, all other treatment groups showed lower count of hypertrophic cells in compare to aged induced group. Figure 2 shows hypertrophic myocardial cells in comparison to normal myocardial cells in heart sections (Table 3).

DISCUSSION

Compared to the D-gal group, the corresponding organ indices in gemfibrozil treatment groups) showed a significant increase in liver and kidney weight. These findings agree with a previous study that confirmed that gemfibrozil administration increases relative and absolute liver weights in male rats,[31] and agree with study done by Liu and his colleagues, which stated that liver index increased by 65% after gemfibrozil treatment in rats when compared with the control group[32] and agree with previously published research that provide evidence for the potential therapeutic of gemfibrozil in clinical practice for mitigating the hepatic renal damages of aging in D-galactose aged mice.[33] Possible explanation for the effect of gemfibrozil is through its role in apoptotic pathways. In previous studies, it had been demonstrated that oxidative stress might lead to apoptosis, which was the main cause of many types of organ damages. Earlier work results also showed that mitochondrial ROS induced the activation of many mitochondrial apoptotic proteins, leading to cellular apoptosis and organ damage.[34]

This match study done by El-Menshawy and her colleagues on albino rats which stated that gemfibrozil have an atiapoptotic effect through an inhibitory action on NF-κB with subsequent inhibition of expression of their target genes.[20]

Regarding heart index, the gemfibrozil treatment group showed a significant decrease in means of heart index compared to the D-galactose group. Studies indicate that oxidative stress and inflammation are closely associated with the progression of myocardial hypertrophy and myocardial infarction. A large amount of evidence of work suggested that oxidative stress can activate nuclear factor-kappa B (NF-kB) in cardiomyocytes, a transcription factor implicated in the regulation of the inflammatory response, which promotes cardiac remodeling and failure.[35]

Compared to the D-galactose age-induced group, the current study showed the significant suppressor effect of gemfibrozil on these proinflammatory mediators. Many research and reviews confirm the current findings and in different organs and different animal models,[36] stated that gemfibrozil inhibits the production of (TNF, IL1β.IL6) by inhibiting of their gene expression in human microglia in a previous study gemfibrozil administration for 2 weeks in male rats along with doxorubicin an anti-cancer drug result in a reduced cardiac level of TNF, IL1β with amelioration of cardiac injury in doxorubicin-

Table 1: Evaluation of gemfibrozil effect on organ index

Group	Mean ± SE		
	Liver Ind.	Kidney Ind.	Heart Ind.
Group 1	8.18 ± 0.19a	0.790 ± 0.02a	0.291 ± 0.01[a]
Group 2	3.46 ± 0.17[b]	0.280 ± 0.03[b]	0.706 ± 0.02[b]
Group 3	6.27 ± 0.17[c]	0.650 ± 0.02[c]	0.448 ± 0.03[c]
Group 4	5.40 ± 0.14[d]	0.496 ± 0.02[d]	0.585 ± 0.01[d]
p-value	0.0001[**#]	0.0001[**#]	0.0001[**#]

Means with different letters differed significantly, SE=Standard Error, significant level at ($p \leq 0.01$). **=highly significant. # *p-value* obtained through One Way ANOVA test (Post hoc Tukey test) Group 1=control group, Group 2=aged induced Group, Group3=vitamin C treatment group, group 4=gemfibrozil treatment group,

Table 2: Effect of gemfibrozil on inflammatory mediators (TNF-α, IL1-β) in heart tissue in all study groups.

Group	*Mean ± SE*	
	TNF-α (ng)	*IL1-β (pg/ml)*
Group 1	42.52 ± 1.22[a]	25.29 ± 0.81[a]
Group 2	341.48 ± 12.79[b]	74.56 ± 0.84[b]
Group 3	78.84 ± 4.12[c]	36.38 ± 1.54[c]
Group 4	131.31 ± 9.79[d]	51.71 ± 1.37[d]
p-value	0.0001[**#]	0.0001[**#]

Means with different letters differed significantly, SE=Standard Error, significant level at ($p \leq 0.01$). **=highly significant. # *p-value* obtained through One Way ANOVA test (Post hoc Tukey test) TNF=Tumor Necrosis Factor, IL1 –β=InterLukin 1 β, IL-6=Interleukin -6, Group 1= control group, Group 2=aged induced Group, Group 3=vitamin C treatment group, Group 4= gemfibrozil treatment group.

Table 3: Means of oxidative stress parameters in heart tissue of all study groups

Group	*Mean ± SE*	
	SOD (pg/mL)	*MDA (ng/mL)*
Group 1	1731.87 ± 26.46[a]	20.54 ± 1.17[a]
Group 2	348.65 ± 16.72[b]	184.97 ± 2.93[b]
Group 3	1019.36 ± 38.18[c]	36.58 ± 2.26[ac]
Group 4	695.25 ± 38.76[d]	78.65 ± 9.14[d]
p-value	0.0001[**#]	0.0001[**#]

Means with different letters differed significantly, SE=Standard Error, significant level at ($p \leq 0.01$). **=highly significant. # P-value obtained through One Way ANOVA test (Post hoc Tukey test) , SOD =Superoxide Dismutase, MDA=Malondehyde, Group 1= control group, Group2=aged induced Group, Group3=vitamin C treatment group, Group4= gemfibrozil treatment group,

induced cardiotoxicity.[37] Gemfibrozil induces the activation of phosphatidylinositol-3 kinase (PI3 Kinase) via PPAR alpha-independent unknown mechanism. PI3 Kinase is a member of growth-supportive survival kinases[38] and PI-3 kinase is responsible for its anti-inflammatory activity. As a result, active PI-3 kinase stimulates gemfibrozil-mediated suppression of proinflammatory molecules.[39] In the present work, after 6 weeks' treatment with gemfibrozil treatment after aging induction is established, results showed a significant rise in SOD enzyme level and a significant reduction in malondehyde level as a product of lipid peroxidation. has also been explored in prior studies. Hakimizadeh and her colleague's study suggested that gemfibrozil treatment improve brain oxidative damage in D-galactose aged mice by increasing SOD levels and reducing Malondehyde levels.[40]

The antioxidant effects of fibrate could be explained by diverse mechanisms that increase the activity and expression of antioxidant enzymes, since PPAR receptors have been found in the promoter regions of many antioxidant genes such as catalase and SOD. So, this modulation could be responsible for gemfibrozil protective effect.[41]

Gemfibrozil antioxidant capacity could also be associated with increase in hepatic glutathione concentration, the major non-enzymatic intracellular defense molecule against ROS.[42] Results of the current study showed a significant reduction in hypertrophic cells in myocardial tissue in treatment groups compared to D-galactose treated group. Gemfibrozil treatment findings are compatible with previous work that confirmed the ability of oral administered gemfibrozil to ameliorate cardiac oxidative stress and hypertrophy in rats.[43] and compatible with the results of earlier work done by Rose *et al.*[44] that indicate the preventive effect of PPAR alpha against the development of pathological cardiac hypertrophy.[44] Mechanisms that may explain the reversing effect of gemfibrozil to the age-associated hypertrophic changes is the antioxidant and anti-inflammatory potential

Age-related oxidative stress has been found to activate ROS-sensitive signaling pathways associated with cardiac hypertrophy and remodeling[45] TNF alpha activation through ROS-dependent pathways,[46] TNF- α -induced hypertrophic responses, including increases in cell size, protein synthesis and further production of ROS and activation of NFκB.[47] ROS also mediated the activation of various hypertrophic signaling kinases, mitogen-activated protein kinases MAPK and the transcription factors nuclear factor-NFκB.[48]

CONCLUSION

Gemfibrozil exerts an anti-aging effect in an accelerated D-galactose-induced mouse model when administered orally after the aged animal model was achieved (treatment effect), since it has a strong anti-inflammatory and antioxidant activity and can potentially reverse age-related cardiac hypertrophic changes. However, further studies are needed to investigate accurate mechanisms. Still, current study findings open the door for promising approaches of gemfibrozil as anti-aging, especially in cardiac aging and related diseases.

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