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Research Article

EFFECT OF FENOFIBRATE ON FREE RADICALS *IN VITRO,* LIPID PEROXIDES, ANTIOXIDANT ENZYMES AND LIVER TRANSAMINASES IN HYPERLIPIDEMIC MICE

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ABSTRACT

In this study, fenofibrate was examined both *in vitro* and *in* vitro. Its antioxidant activity *in vitro* was screened using DPPH assay; and specific antioxidant activity was observed using nitric oxide (NO•) assay, hydroxyl radical (•OH) assay and TBARS assay. The same substance was administered in mice to observe its antioxidant enzyme – GSH) and catalase - induction, inhibition of serum transaminases –SGPT and SGOT, and lipid lowering activities. It was observed *in vitro* that DPPH (IC_{50} > 0.38 mg/mL), NO• (IC_{50} = 22.81 µg/mL) and lipid peroxidation (IC_{50} = 30.37 mg/mL) was inhibited but not •OH (SC_{50} < 0.05 mg/mL). *In vivo* experimentation for lipid peroxidation showed that TBARS concentration was decreased by 24.79% while increasing concentrations of both GSH and catalase. Serum transaminases were reduced by 3.65% and 8.93%, respectively. Lipid profiling showed a decline in triglycerides and low density lipoproteins by 54.87% and 16.67%, correspondingly; while the amount of high density lipoproteins was augmented by 45.13%. Fenofibrate scavenges of free radicals, stimulates •OH production that may increase body's defense against pathogens, boosts liver antioxidant enzyme against free radicals, and lowers serum transaminases. The results strongly suggest that fenofibrate, aside from its lipid-lowering activity may also provide antioxidant defenses.

Keywords: fenofibrate, antioxidant, antioxidant enzymes, serum transaminases, lipid profile.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. One of the known types of PPAR is PPARα. It has been recognized to regulate lipid metabolism in liver, heart, kidney and muscles. Its exogenous agonist, fibrates, lowers the plasma triglycerides effectively and is used in hyperlipidemia treatment[1]. According to the Rational Assessment of Drugs and Research, fibrates are used first in line in patients with mixed dyslipidemia when an elevated concentration of triglycerides is the principal abnormality. This has been backed up by Beltowski, J. and colleagues (2002) that administration of 300 mg/kg/day (0.05%) fenofibrate in Wistar rat diet decreased their plasma MDA by up to 87.1%.

Fenofibrate (propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl] phenoxy}-2-methylpropanoate) is a member of fibrate class of lipid-modifying drugs and is a third generation fibric acid derivative. Fenofibric acid, its active form, is easily achieved by rapid hydrolysis orally. It specifically increases lipoprotein lipase activity which limits the availability of fatty acids that are needed for the formation of triglycerides. It is also known to stimulate reverse cholesterol transport and suppress HMG-CoA reductase activity. As a PPAR-α agonist, it plays an important role in many vascular diseases such as

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diabetes mellitus (DM), hypertension and coronary heart disease. But aside from this, it is also used as drug for lipid-related diseases like dyslipidemia and hypercholesterolemia. Another important activity of PPAR-α is that it produces a notation of an antioxidant capacity by lowering malondialdehyde (MDA), an indicator of lipid peroxidation, and by stimulating the expression of superoxide dismutase (SOD), a major antioxidant enzyme[2]. In a study done by Watts, G.F. & Staels, B., they reported that fenofibrate induced an increased activity of endothelial nitric oxide synthase (eNOS), which forms nitric oxide[3].

In patients suffering from both DM1 and DM2, hyperhomocysteinemia is an independent risk factor for macroangiopathy and mortality[4]. They experience auto-oxidation of homocysteine (Hcy) that generates numerous reactive oxygen species (ROS). These ROS may then initiate lipid peroxidation in cellular membranes which are deemed responsible for endothelial injury that subsequently leads to reduction of vascular nitric oxide (NO•) production[5]. Mechanisms of how Fenofibrate increases the Hcy levels and whether it may have any adverse effect are not well understood.

Exposure of low-density lipoprotein (LDLs) to reactive oxygen species plays an important role in the biological system. Oxidized LDLs can: decrease NO• availability, stimulate inflammatory response of macrophages, activate migration and proliferation of vascular smooth muscle cells and induce immune response[1].

This study aims to prove that Fenofibrate can not only lessen triglycerides in the serum, but also act as an antioxidant therefore decreasing lipid peroxidation which leads to decreased oxidized LDL. In the present status of antioxidant studies[6] of fenofibrate, there is a lack of literature that suggests the specific activity of fenofibrate on different reactive oxygen species. Serum lipid profile will be measured to observe changes in serum values of triglycerides (TG), LDL, and high-density lipoprotein (HDL). Antioxidant activity will be measure both *in vivo* and *in vitro*. *In vitro* analyses include 1'-diphenyl-2-picrylhydrazyl (DPPH) assay, hydroxyl radical (•OH) assay, nitric oxide (NO•) assay and TBARS assay. *In vivo* analysis will measure serum lipid peroxidation using TBARS assay, hepatic enzymes reduced glutathione (GSH) and catalase (CAT), and serum transaminases: serum glutamate pyruvic transaminases (SGPT) and serum glutamate oxaloacetic transaminases (SGOT).

Material and Methods

2.1. Chemicals

The antioxidant and hyperlipidemic effects of fenofibrate were tested both on rats with Triton X-100-induced hyperlipidemia and *in vitro*. For *in vivo* analysis, 20 female Sprague-Dawley rats weighing 100-150g were purchased from the Food and Drug Administration (FDA) which were used as experimental animals. Hyperlipidemia was induced by intraperitioneal injection of Triton X-100 (100 mg/kg BW). Serum was analyzed for concentrations of transaminases and lipids using enzymatic methods. Homogenized liver was used for TBARS concentration analysis and hepatic enzyme activities. These activities were analyzed to determine the hepatoprotective, anti-lipid peroxidative and antioxidant effects of Fenofibrate (Sigma-Aldrich, Singapore). Solvents and other chemicals used were bought from Golden Bat Inc. (Quezon City, Philippines).

2.2. In vitro DPPH Scavenging

The procedure used was similar to that previously described Green RJ[7]. DPPH assay is a general assay for antioxidant screening. It measures the decrease in purple DPPH after the addition of an antioxidant which consequently loses color. The assay was carried out by adding 150 μL of different concentrations of fenofibrate to 3 mL of $6x10^{-5}$ M DPPH solution in methanol then incubating it at room temperature in a dark location for 30 min. The reaction mixture was read at 517 nm. Gallic acid was used as positive standard.

2.3. In vitro •OH Scavenging by Fenton Assay

The procedure used was similar to that preciously described Winterbourn[8]. The scavenging of hydroxyl radical was performed using the Fenton assay. This assay measures the changes in the amount of •OH radicals after the addition of the antioxidant. A decrease in absorbance in addition of an antioxidant indicates the scavenging of •OH radicals in the reaction mixture[9]. The Fenton reagent, which consists of 0.1 mM $FeCl_3$, 1.5% (w/v) H_2O_2 and 0.0029% (w/v) EDTA, was added to 0.6 mL of different concentrations of fenofibrate. The absorbance of the reaction mixture was read at 288 nm to measure the reduction in numbers of hydroxyl radical by fenofibrate.

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2.4. In vitro NO• Scavenging

The procedure used was similar to that preciously described[10]. The nitric oxide assay was used to determine the scavenging activity of NO• scavenging of fenofibrate. In a tube, 10 mM sodium nitroprusside (SNP), PBS (pH 7.4), and various concentration of fenofibrate was added, totaling to 3 mL. This mixture was incubated for 150 min at 25°C. After incubation, 1 mL of 0.33% sulfanilamide (in 20% acetic acid) was added to 0.5 mL of the reaction mixture. This was allowed to stand for 5 min. After 5 min, 1 mL of 0.1% w/v napthylethylenediamine dihydrochloride (NED) was added and again incubated for 30 min at the same temperature. The pink chromophore produced through the diazotization of nitrite ions with sulfanilamide and its consequent coupling with NED was measured at 540 nm.

2.5. Animal Protocol

The animals were divided into three groups of four rats each treated for two weeks as show in Table A.1. During the two-week experimental period, the animals had *ad libitum* access to both rodent chow pellets (Purina Mills) and distilled water except for fasting period (12 hours overnight) prior to hyperlipidemia induction and blood extraction. The rats were acclimatized for 7 days under room temp of 25-30°C and 80% humidity with 12-hours light-dark cycle. The rats were handled according to the rules and regulations of the University of Santo Tomas (UST) Institutional Animal Care and Use Committee (IUCAC).Triton X-100 (100 mg/kg BW)[11] and fenofibrate (10 mg/kg BW) concentrations followed the method of Keshetty, *et al.*[12]. Fenofibrate and NSS were administered orally by gavage. Blood extraction was administered using tail clipping method.

2.6. Lipid peroxidation (TBARS assay)

Previously described by Oyinbo, *et al.*[13]. TBARS assay determines the amount of lipid peroxidation by measuring its end-product malondialdehyde. This pink colored aldehyde is measured and decrease in the absorbance signifies inhibition of lipid peroxidation[14]. For this analysis, a volume of 0.6 mL of 0.67% (w/v) thiobarbituric acid (TBA) and 0.75 mL of 8% (w/v) trichloroacetic acid (TCA) was added to 0.15 mL liver homogenate. The rat liver in the control was used as reference.

For *in vitro* analysis[15], the same volumes of TBA, TCA and liver homogenate were added to 0.15 mL of Fenofibrate. The reaction mixture was heated in a 100°C water bath for 15 min, followed by a centrifugation at 13000g. The pink chromogen formed was measured at 532 nm.

2.7. Hepatic Antioxidant: Estimation of Reduced Glutathione (GSH)

Reduced glutathione was measured to observe if fenofibrate affects its release. GSH is one of the major natural antioxidant defense of the body. The method used in quantifying GSH in the liver is as previously described Kaur, *et al.*[16]. An aliquot of 0.5 mL of liver homogenate was precipitated with 0.5 mL of 4% (w/v) sulphosalicylic acid. The samples were kept at -4.0°C for 1 hr followed by centrifugation at 1200g for 15 min. The assay mixture was prepared by combining 0.1 mL aliquot of fenofibrate, 2.7 mL phosphate buffer and 0.2 mL DTNB. The absorbance was read at 412 nm.

2.8. Hepatic Antioxidant: Catalase Activity

Catalase, including GSH, is classified as antioxidant enzymes. It acts by directly decomposing hydrogen peroxide to ground state O_2. The amount of CAT in the liver was measured using a method previously described Kaur, *et al.*[16]. Mixture of 1.95 mL of phosphate buffer, 1.0 mL H_2O_2 and liver homogenate was mixed in a tube. After mixing, the change in absorbance was read at 240 to calculate the CAT activity.

2.9. Analysis of serum lipid profile and serum transaminases

Triglyceride, LDL and (HDL) – for serum lipid profile; serum glutamate pyruvic transaminases and serum glutamate oxaloacetic transaminases – for serum transaminases were analyzed by SIM Clinical Laboratory (Manila, Philippines). SIM Clinical Laboratory (TIN no. 000-333-374-000; DTI Business permit certificate no. 00116763) is a Center for Health Development-licensed laboratory accredited by the Department of Health.

2.10. Statistical analysis

The results gathered in this study will be expressed as mean ± SD. One-way analysis of variance (ANOVA) and Post-hoc analysis for multiple comparisons were used to determine if there are significant differences between the concentrations fenofibrate and $p < 0.05$ was regarded as statistically significant.

Results and Discussions

3.1. In vitro analysis

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Fenofibrate was tested for its antioxidant activity *in vitro* through: DPPH assay, nitric oxide assay, hydroxyl radical assay and TBARS assay.

3.1.1. DPPH assay

DPPH is a stable and well characterized solid radical source, which is traditionally used to determine the free radical scavenging activity of a potential antioxidant. This assay is based on the ability of DPPH assay to decolorize in the presence of antioxidants. Antioxidants reduce DPPH radical to 1, 1 – diphenyl – 2 – picryl hydrazine, a colorless compound. Decolorized DPPH can be quantitatively measured from the changes in absorbance, wherein a decrease in absorbance correlated with higher DPPH radical- scavenging activity of the antioxidant.

Figure 1 shows the activity of fenofibrate against DPPH radical, with the maximum percentage of control of 87.2%. Median inhibitory concentration (IC_{50}) of fenofibrate against DPPH was computed at >0.38 mg/mL. This represents the H^+- donating ability of fenofibrate. The concentration of the purple DPPH radical decreases as its odd electron is paired with hydrogen from the antioxidant. Thus, results showed the general antioxidant ability of fenofibrate.

3.1.2. Fenton Assay

Fenton assay is based on the ability of the antioxidant to scavenge the highly –reactive •OH. This radical can be generated via Fenton reaction where H_2O_2 reacts with Fe^{+2} bound to EDTA to yield •OH. In this assay, a decrease in absorbance indicates the ability of the antioxidant to scavenge the hydroxyl radical present in the reaction mixture.

Figure 2 shows the activity of fenofibrate on the highly – reactive •OH. Media stimulatory concentration (SC_{50}) of fenofibrate on hydroxyl radical was <0.05 mg/mL. The sample showed a stimulatory effect on hydroxyl radical at all concentration. Thus, as shown by the increased percentage control as the concentration increase, fenofibrate exhibits a pro-oxidant effect.This activity may be compared with the known antioxidant ascorbic acid, which produces •OH on higher concentrations. But aside from this, stimulation of •OH radicals may be of help for our body\s defense against pathogenic substances. It is known that macrophage and granulocytes releases this free radical to battle against bacteria that may cause infection[17].

3.1.3. Nitric Oxide

In nitric oxide assay, NO• is generated from the breakdown of sodium nitroprusside (SNP) which when reacted to O_2 produces nitrite ions (NO_2^-). Nitrite ions reacted with sulfanilamide and NED produces pink solution which can be measured at 540 nm.

As shown in Figure 3, NO• was reduced by the addition of fenofibrate but not dose-dependently, with a maximum percentage of 45.84%. Median inhibitory concentration of fenofibrate against NO• was computed at 22.81 µg/mL. NO• is a reactive nitrogen specie (RNS), which reacts with superoxide radical (O_2•$^-$) to generate the cytotoxic peroxynitrite ($ONOO^-$). Addition of peroxynitrite to cell leads to rapid protonation followed by depletion of –SH groups, oxidation and nitration of lipids, DNA strand breakage, and nitration and deamination of DNA bases. Thus, these deleterious events can be prevented by the scavenging of NO•. In addition, excessive production of NO• may also cause tissue injury and vascular collapse. On the other hand, the reported induction of eNOS by fenofibrate in cell culture cannot be ignored[3]. This may suggest the NO• modulation activity of fenofibrate.

3.1.4. TBARS assay

Thiobarbituric acid reactive substances (TBARS) assay is a well-established method for screening and monitoring peroxidation of lipids. It is an assay where malondialdehyde (MDA) forms a 1:2 adduct with TBA to form a pink chromogen which can be measured at 532 nm. An increase in the intensity of the pink chromogen formed may correlate with the oxidative rancidity of the lipids.

Fenofibrate had a maximum percentage control of 11.18% on TBARS assay as shown in Figure 4. The IC_{50} of fenofibrate against lipid peroxidation was computed at 30.37 mg/mL. This remarkably low value indicates the fenofibrate effectively protects lipoproteins from lipid peroxidation. Lipid peroxidation is a free radical-mediated, particularly, a hydroxyl radical-mediated degradative process where the double bonds of polyunsaturated fatty acids rearrange, resulting to reduced membrane fluidity.

3.2. In vivo analysis

The antioxidant and hypolipidemic effects of fenofibrate on rats with Triton X-100- induced hyperlipidemia were determined by obtaining their liver and sera.

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Table 1 show the food and drug administration for the *in vivo* study.

Group	Feed	Treatment
I (Negative Control)	Rodent chow + NSS	No Fenofibrate
II (Positive Control)	Rodent chow + Triton X-100	No Fenofibrate
III (Experimental)	Rodent chow + Triton X-100	With Fenofibrate

Figure 1. DPPH radical scavenging activity of fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with control.

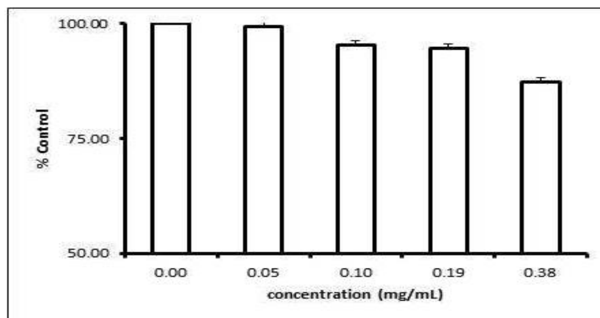

Figure 2. Activity of fenofibrate on hydroxyl radical. Values (n=2) represent mean ± SD, and *p<0.05 compared with control.

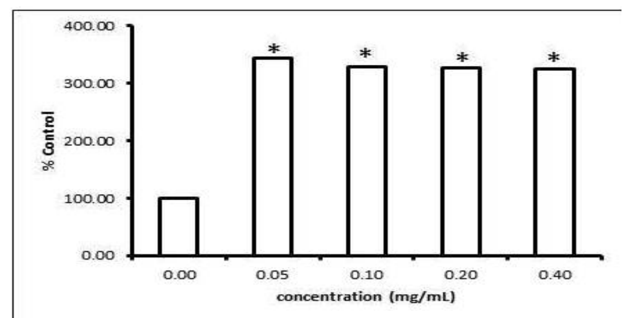

Figure 3. Scavenging activity of fenofibrate against nitric oxide. Values (n=2) represent mean ± SD, and *p<0.05 compared with control.

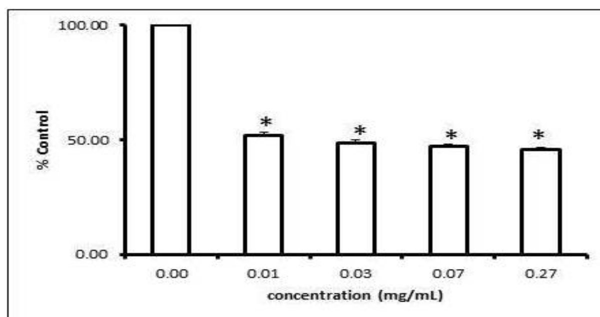

Figure 4. Inhibition of lipid peroxidation by fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with control.

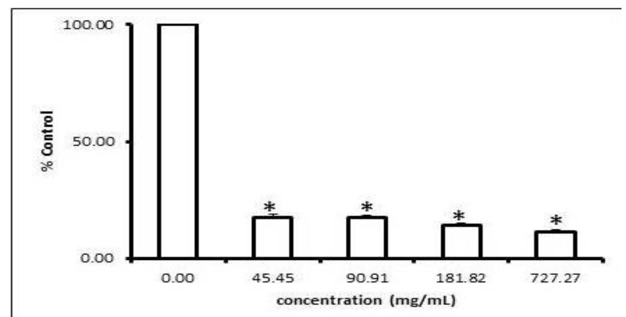

Figure 5. Prevention of lipid peroxidation by fenofibrate on hyperlipidemic livers of rats (n=4). Values (n=2) represent mean ± SD, and *p<0.05 compared with the Triton group.

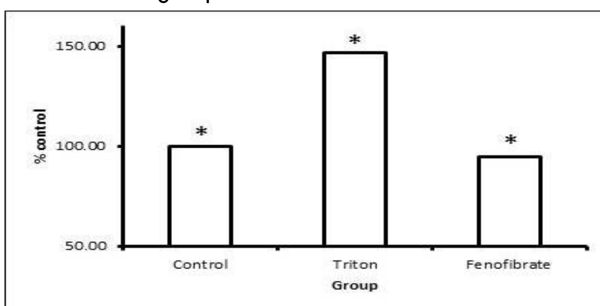

Figure 6. Increase in the levels of GSH on livers of hyperlipidemic rats (n=4) by fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with the Triton group.

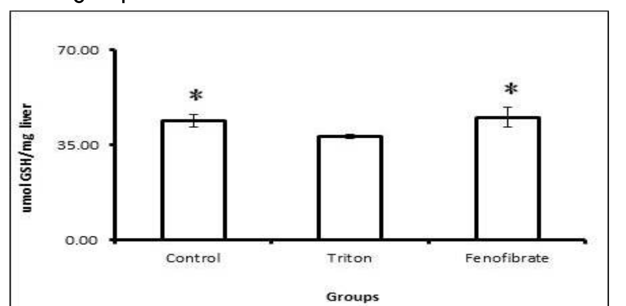

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Figure 7. Increase in the levels of CAT on livers of hyperlipidemic rats (n=4) by fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with the Triton group.

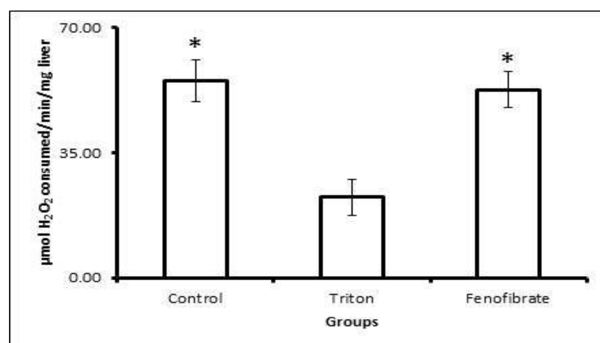


Figure 8. Decreased GOT activity on serum of hyperlipidemic rats (n=4) by fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with the Triton group.

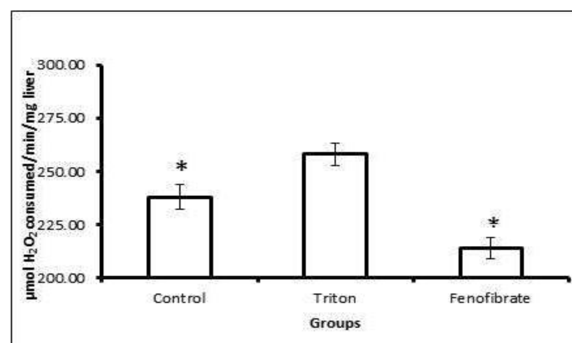
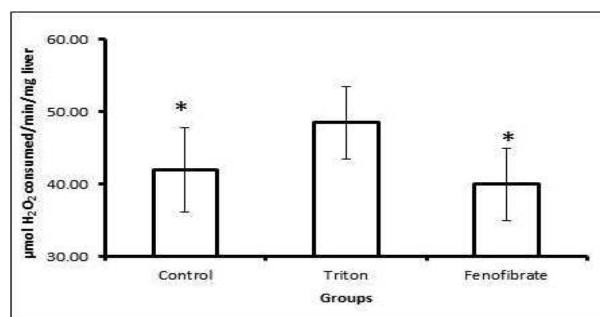


Figure 9. Decreased GPT activity on serum of hyperlipidemic rats (n=4) by fenofibrate. Values (n=2) represent mean ± SD, and *p<0.05 compared with the Triton group.



3.2.1. TBARS assay

For the *in vivo* analysis, rat liver homogenate was used for the measurement of lipid peroxide levels. As shown in Figure 5, hyperlipidemic livers had an increase of 53.44% in TBARS concentration compared to the control. Triton X-100, a non-ionic detergent, interferes with the normal rate of lipid removal from the blood. It also has the capability of inhibiting lipoprotein lipase, thus, its elevated levels were confined to the blood of the Triton-induced animal.

Fenofibrate significantly decreased TBARS concentration by 24.79% compared to the Triton group. This result supports the claims that Fenofibrate makes apo-B containing lipoproteins more resistant to oxidative modifications[18].

3.2.2. Hepatic antioxidants: Reduced glutathione and catalase

Glutathione reductase and CAT are two of the antioxidant enzymes. The former maintains appropriate levels of GSH, while the later directly decomposes hydrogen peroxide to ground state O_2. Livers of hyperlipidemic rats had lower activities of these enzymes, but treatment of fenofibrate, as shown in Figure 6, improved the hepatic antioxidant status compared to the Triton group.

Reduced glutathione, an antioxidant produced by glutathione reductase, occurs naturally in all human cells. It is an antioxidant with an important role in detoxification of reactive oxygen species. In the presence of glutathione reductase, two moles of GSH can convert cellular hydrogen peroxide to water. Intracellular depletion of GSH ultimately results in cell death[19]. Figure 6 shows the elevated levels of GSH in the livers of hyperlipidemic rats with treatment of fenofibrate.

Catalase, on the other hand, is an antioxidant enzyme present in most aerobic cells. It is involved in the detoxification of hydrogen peroxide by catalyzing its conversion to molecular oxygen and water[20]. Inadequate removal of reactive oxygen species results in oxidative stress which may cause damage to biological macromolecules. Figure 7 shows increased CAT by treatment of fenofibrate.

The increased CAT activity may be related to the NO• scavenging of fenofibrate. According to Halliwell and Gutteridge[21], NO• reversibly binds to the haems of CAT. Once this occurs, CAT activity declines. But since fenofibrate inhibits NO•, less NO• binds to CAT. This in turn increases the enzymatic activity of CAT. This shows the relation of the free

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radical scavenging activity of fenofibrate to its ability to improve the enzymatic antioxidant defense status *in vivo*.

3.2.3. Serum transaminases: SGOT and SGPT

Glutamate oxaloacetate transaminases (GOT) and glutamate pyruvate transaminases (GPT) are both found in the liver, with GPT as the more specific hepatic enzyme. Its leakage and elevated levels in the blood indicates damage of the source organ[22]. This justifies why activities of transaminases were elevated in the sera of hyperlipidemic rats. Figure 8 showed that fenofibrate treatments reduced the SGOT activity by 8.93%. The same reducing trend was observed with the SGPT activity at 3.65% as shown in Figure 9.

Decrease serum transaminases and increased hepatic enzymes both characterize hepatoprotection. Membrane integrity is enhanced with the presence of antioxidant defense enzymes. With this, transaminases, the indicators of liver and other organ damage, are prevented from leaking into the blood stream.

3.2.4. Serum lipids

Serum TGs and LDL-C (Cholesterol) increased while HDL-C (Cholesterol) levels decreased after hyperlipidemia was induced in rats. After the two-week experimental period, fenofibrate improved the lipid profile of rats.

Administration of 10 mg/kg BW fenofibrate on hyperlipidemic rats significantly increased HDL-C by 45.13% and significantly decreased TG and LDL-C by 54.87% and 16.76%, respectively.

The remarkable decrease in the TG levels by fenofibrate supports the literature stating that it increases the expression of genes for lipoprotein lipase, and decreases the expression of apolipoprotein CIII[23]. Apoliproprotein CIII is a known potent inhibitor of lipoprotein lipase while apolipoprotein CII activates the same enzyme. An imbalance in apo CIII/CII ratio due to increase in plasma apolipoprotein CIII may cause inactivation of lipoprotein lipase[24].

Conclusion

The results suggest that fenofibrate inhibits DPPH, NO• but not •OH. It also reduces TBARS concentration both *in vitro* and *in vivo*. In hyperlipidemic rats, it increases the concentrations of GSH, enhances the activity of CAT, reduces the activity of serum transaminases, and improves lipid profile.

Therefore, fenofibrate is a drug that can also be used as an antioxidant that promotes protection of the liver in addition to its major lowering of triglycerides.

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