PPARα-dependent Increase of Mouse Urine Output by Gemfibrozil and Fenofibrate

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PPARα-dependent Increase of Mouse Urine Output by Gemfibrozil and Fenofibrate

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Abstract

While gemfibrozil and fenofibrate are prescribed for anti-dyslipidemia treatment, a rational basis for the use of these drugs for treatment of dyslipidemia with concurrent metabolic syndrome has not been established. In this study, wild-type and \textit{Ppara-null} mice were fed gemfibrozil- or fenofibrate-containing diets for 14 days. 24-hour urine output was monitored, and urine, serum, liver and kidney tissues were subjected to toxicity assessment. A two month challenge followed by a two week wash-out was performed for gemfibrozil to determine urine output and the potential toxicicity. A therapeutically equivalent dose of gemfibrozil was more effective than fenofibrate in increasing urine output. This regulatory effect was not observed in \textit{Ppara-null} mice. In contrast, hepatomegaly induced by fenofibrate was more pronounced than that of gemfibrozil. No significant toxicity was observed in liver or kidney in the 2-month treatment with gemfibrozil. These data demonstrated \textit{PPAR\alpha} mediates the increased urine output by fibrates. Considering the relative action on hepatomegaly and the regulatory effect on urine output, gemfibrozil may be the preferable drug to increase urine output. These results revealed a new pharmacodynamic effect of clinically-prescribed \textit{PPAR\alpha} agonists and suggested the potential value of gemfibrozil in modification of blood pressure.

Keywords

Gemfibrozil; urine output; \textit{PPAR\alpha}; fenofibrate
Introduction

Metabolic syndrome (MS) is characterized by high serum triglyceride (TG), elevated blood pressure, abdominal obesity, elevated fasting plasma glucose and low high-density lipoprotein-cholesterol (HDL-C). In USA, the unadjusted MS prevalence was reported to be 21.8%, which increased to 43.5% and 42.0% for participants aged 60 through 69 years and those above 70 years (Ford et al. 2002). The Lifestyle Interventions and Independence for Elders Study reported a prevalence of 49.8% in a population of sedentary adults aged 70 to 89 (Botoseneanu et al. 2015). Concurrent metabolic diseases trigger more therapeutic challenges for clinicians. Approximately 50% of patients with essential hypertension, treated and untreated, appear to be insulin resistant. And CVD risk factors in these patients are greatly accentuated (Lima et al. 2009). Hyperlipidemia and hypertension are both included in the diagnostic criteria of MS, so hypertriglyceridemia patients with concurrent hypertension are at more risk to develop MS.

Peroxisome proliferator-activated receptor alpha (PPARα) regulates genes involved in the metabolism of lipids, glucose, and amino acids (Peters et al. 2005). Links between PPARα and the metabolic diseases including diabetes, obesity, dyslipidemia have been suggested. This raises an untested hypothesis that PPARα activation may be preventive for the progression of metabolic diseases. In addition to the studies on the treatment of hyperlipidemia, the differential effects of clinical PPARα agonists on glucose metabolism were reported (Avogaro et al. 1995; Liu et al. 2011; Noguchi et al. 2011; Sheu et al. 1993). In a mouse model, sodium excretion was
faster in Ppara-null mice than in wild type mice (4.31+/E1.11 vs 0.77+/E0.31) in
response to an acute NaCl-load. This effect became less in amiloride-treated mice
(3.4- vs 15.5-fold) (Obihan and Oyekan 2008). However, no reports were found
about regulation of urine output by clinical PPARα agonists directly.

Fibrate drugs are PPARα agonist widely used for the treatment of
hypertriglyceridemia and mixed hyperlipidemia associated with atherosclerosis
(Fievet and Staels 2009). They promote TG and very low-density lipoprotein (VLDL)
catabolism, which reduces the risk of cardiovascular events. However,
gemfibrozil-mediated improvement in cardiovascular disease (CVD) risk might not be
the result of its effects on HDL-C. The cardiovascular benefits of gemfibrozil were
supposed to be due to the effects on metabolic processes not reflected by its
modification of lipid profiles (Asztalos et al. 2008). The role of fibrates in control of
blood pressure, in addition to the classic pharmacodynamic effects on triglyceride
metabolism, is of great interest.

The effect of fibrate drugs on blood pressure associated has been reported. In
Sprague Dawley rats receiving Ang II infusion, fenofibrate significantly reduced the
systolic blood pressure (SBP), suggesting a role in the control of hypertension (Diep
et al. 2004). A combination of fenofibrate and rosiglitazone in a rat model was
reported to lead to attenuation of hypertension (Ciuceis et al. 2007). In type II diabetic
subjects with left ventricular diastolic dysfunction, fenofibrate and coenzyme Q10
interactively lowered 24-h SBP (Chew et al. 2008). In another study using a rat model,
chronic treatment with the PPARα agonist clofibrate treatment reduced salt-dependent
arterial pressure (Williams et al. 2005). For bezafibrate, a significant reduction of SBP and diastolic blood pressure was reported in patients with hypertension and hyperlipidemia (Kim et al. 2003). Hypertension is one of the most important markers in the clinic to diagnose MS cases. Diuretics belong to the first line agents for treatment of hypertension in the clinic and are the basic agents for combination with the other drugs. Thus regulation of urine output is important for both patients with hypertension and those of MS. However, an association between attenuation of hypertension and urine output regulation by fibrates has not been suggested.

In this study, wild-type and Ppara-null mice were administered gemfibrozil and fenofibrate to evaluate the effects on urine output and potential kidney toxicity. Gemfibrozil was found to increase urine output with less toxicity compared with fenofibrate, which was dependent on PPARα. These data suggest PPARα's role in regulating urine output and the potential use of gemfibrozil rather than fenofibrate to treat hypertriglyceridemic-hypertensive patients.
Materials and methods

Chemicals and reagents

Gemfibrozil was obtained from Hunan Qianjin Xiangjiang Pharmaceutical Co. Ltd (Zhuzhou, China). Fenofibrate was purchased from Shangqiu Chemry Chemicals Co. Ltd (Shangqiu, China). All the ELISA kits were purchased from B&D Systems (Shanghai, China). The reverse transcription kit was product by Thermo Fisher (PA, USA). The LightCycle 480 SYBR Green I Master Mix was purchased from Roche. Purified water was freshly prepared using a Millipore Elix (MA, USA) system. All the other chemicals were of the highest grade available from commercial sources.

Animals and treatment

WT and Pparα-null mice on the 129/Sv background, described previously (Lee et al. 1995; Yang et al. 2008), were gifted by Dr. Gonzalez at NCI, NIH, USA. All mice were maintained in the animal center of Medical School of Ningbo University, under a standard 12 h light/12 h dark cycle with free access to water and a commercial diet. All procedures were performed in accordance with Institute of Laboratory Animal Resource Guidelines and protocols approved by the Medical School of Ningbo University. Male five to seven-week-old mice were acclimated to a commercial purified diet for seven days before the experiment. WT mice were fed a gemfibrozil (0.375%, 0.75% and 1.5%) and fenofibrate (0.075%, 0.15%, and 0.3%) diet (WT-L, WT-M and WT-H) respectively for 14 days where the dosage was designed based on their relative doses in the clinic. Considering the renal function response and urine volumes observed in the WT mice, the Pparα-null mice were fed high gemfibrozil
and fenofibrate diets only (KO-H). The control groups for both mouse lines and agents were treated with purified diet only (WT-C and KO-C). All the mice were placed into metabowls on day 0, 7 and 14 during the treatment. Each mouse was weighed and food intake measured over 24 h. The urine samples over 24 h were collected using metabowls. The volumes were normalized by body mass and expressed as mL/10 gm. Just before sacrifice by cervical dislocation, blood as much as possible was taken by retro-orbital bleeding for toxicity assay under CO₂ narcosis.

Because of the strong regulation, a sub-chronic experiment was performed for gemfibrozil, to find potential toxic endpoints and the reversibility of urine output regulation. Specifically, five WT and five Ppara-null mice were fed a high

gemfibrozil diet for two months followed by a two week clearance. Urine output was measured on day 0, 30, 60 after dosing (n=five). Two mice from each group were killed on day 60. Urine output of the remaining three mice in both groups was measured seven and fourteen days after diet replacement, following which the mice were killed.

At the end of each experiment, all mice were weighed and killed after blood

collection. Renal and liver tissues were harvested and weighed to calculate organ/body weight ratio. A section of renal and liver tissues were excised and immediately fixed in 10% neutral buffered formalin. The remaining tissues were flash frozen in liquid nitrogen. All urine, serum and tissue samples were stored at -80°C pending analysis.

Analysis of urine indicators
Glucose, hematuria, protein, bilirubin, urobilinogen and ketone body in the urine were measured by clinical standard methods, to assay the potential toxic effect of urine output regulation.

**Biochemical assessment of serum samples**

The classic hepatotoxicity indicators alanine transaminase (ALT), aspartate transaminase (AST), creatinine and blood urea nitrogen (BUN) were assayed by Spectra Max M5 (CA, USA), using an end-point method or a dynamic method. Antidiuretic hormone (ADH) and aldosterone (ALD) levels were determined using ELISA kits (Shanghai, China). In the above determination, 2 µL sample was added for each measurement. An aliquot of 50 µL of the remaining sample was diluted (1:10) for measurement of K⁺, Na⁺, Ca²⁺ and Cl⁻ by standard methods in the Affiliated Hospital of Medical College of Ningbo University.

**Histopathological analysis**

Fixed kidney tissues were subject to dehydration in a serial concentration of alcohol and xylene for paraffin embedding. Four-micrometer serial sections were cut and stained with hematoxylin and eosin, followed by histological examination under an Olympus BX41 light microscope.

**Quantitative polymerase chain Reaction (Q-PCR) analysis**

Expression of mRNAs encoding kidney transporters involved in the regulation of urine output, including aquaporins (AQP), sodium-chlorine cotransporter (NCC), sodium-hydrogen exchanger (NHE), sodium-potassium-chloride cotransporter (NKCC) were measured. Total mRNA was extracted from approximately 20 mg
frozen kidney tissues, using TRIzol reagent (Carlsbad, CA). Total RNA levels were
determined by use of Nano Drop 2000 (Roche, USA). Reverse transcription was
performed using standard procedures where first-strand cDNA was generated from 1
µg total mRNA with a SuperScript II Reverse Transcriptase kit. The primers extracted
from public database (http://mouseprimerdepot.nci.nih.gov/) were tested for
specificity and used in the PCR amplification. Each 10 µL PCR system contained 1
µL total cDNA, LightCycle 480 SYBR Green I Master Mix (FastStart Taq DNA
Polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and MgCl$_2$) 5 µL,
forward primer 0.2 µL, reverse primer 0.2 µL, nuclease-free water 3.6 µL. The
fluorescence signals were detected at the end of each cycle. Specific mRNA levels
were normalized to 18S rRNA and expressed as fold change equivalent to that of
WT-C.

Statistics

All data were expressed as the mean ± SD. SPSS 13.0 for Windows (Chicago,
USA) was used for the data analysis. The results following treatment in both WT and
Ppara-null mice were compared with their baseline levels or those in their control
groups respectively. A two-tailed, independent-sample $t$-test was used to analyze the
results from Ppara-null mice, and the organ indices or other indicators between
wild-type mice and Ppara-null mice. One-way ANOVA followed by Fisher’s least
significant difference post hoc tests was used for multiple comparisons of data from
wild-type mice and those time-related alterations. A difference was considered
significant when the $p$ values were less than 0.05 ($p<0.05$).
Results

Urine output regulated by gemfibrozil and fenofibrate

Compared with the WT-C group, WT mice dosed with low, medium, high gemfibrozil exhibited increased urine output, with the volume of the urine output of the WT-H group about 6 times of that of WT-C group (Fig. 1A, \( p<0.05 \)). Compared with the WT-C group, no difference was found in KO-C group (Fig.1A and B). No significant difference was found between the KO-C mice and KO-H mice (Fig. 1B, \( p>0.05 \)), indicating a critical role for PPARα in regulation of urine output.

With fenofibrate, the urine output of WT-H mice doubled in comparison with that of the WT-C mice. This increase was much smaller than that of gemfibrozil (2 times vs 6 times). Similar to the gemfibrozil, there was no increase in urine output in the KO-H mice compared with the KO-C mice. These results further demonstrated a role of PPARα in the regulation of urine output, with gemfibrozil more effective than fenofibrate in increasing urine output in the mouse model.

Toxic responses of liver and kidney

The liver/body weight ratios increased significantly in the three groups treated with gemfibrozil and fenofibrate compared with the WT-C group. However, it was evident that the liver/body weight ratio of the WT-H mice treated with gemfibrozil diet (7.2±0.4%) was much smaller than those treated with fenofibrate (10.1±0.9%) (Fig. 2A and B, \( p<0.05 \)). The liver/body weight ratio of the KO mice treated with high gemfibrozil and fenofibrate diets were significantly different compared with their control groups (Fig. 2C and D, \( p>0.05 \)). Biochemically, no changes in AST or ALT
were found with either fibrate treatment (Supplementary Figure S1A-D), which correlated with the negative pathological result in the liver tissues (data not shown).

For the weight of kidney tissues, no difference was noted between control mice and treated mice (either WT or Ppara-null) for either fibrate (Fig. 2A-D). In the clinic, increase of BUN and creatinine often suggest nephropathy. In the 14-day experiment, no increase in creatinine was found among those WT and KO groups. However a significant difference of BUN was detected between WT-H and WT-C (Fig. 3A and B, \( p < 0.05 \)). No histopathological changes in kidney tissues were observed in either mouse line after treatment with the gemfibrozil diets (Fig. 3C-H). For the fenofibrate experiment, the kidney responses were similar as those of gemfibrozil (Supplementary Figure S2). The urine toxicity indicators glucose, hematuria, protein, bilirubin, urobilinogen and ketone body were analyzed by clinical method after gemfibrozil and fenofibrate treatments, but none were changed, indicating no toxicity was associated with increased urine production (data not shown). For the two fibrate treatment, the biochemistry, pathology and urine analysis results correlated well and indicated a tolerance for gemfibrozil and fenofibrate in the mouse kidney.

**Serum homeostasis and mechanism exploration**

No differences were observed for \( \text{Na}^+ \), \( \text{K}^+ \), \( \text{Ca}^{2+} \) and \( \text{Cl}^- \), between the WT or/and Ppara-null mice after gemfibrozil treatment (Fig. 4A-D). Difference was significant in Ppara-null mice for \( \text{Na}^+ \) and \( \text{Cl}^- \) except for \( \text{K}^+ \). Additionally, modification in WT mice did not correlate with either the urine output or the serum ADH and ALD (Fig. 5A and B). Similar negative responses were found with fenofibrate treatment.
AQPs are a group of water channels involving water transportation. *Aqp 1-3* and the ions transporter *Nhe, Ncc* and *Nkcc* mRNAs known to be correlated with urine output were measured. However, no differences were found between the WT mice treated with gemfibrozil and the control group. Although some were down-regulated in *Ppara*-null mice, the decrease were less than 2 fold, thus indicating no biological significance (Fig. 5C and D). These data indicated that the mechanism of gemfibrozil alteration of urine output might not involve modification of transporter expression in the kidney.

**Pharmacodynamic and toxic analysis of two-month treatment**

In the 2-month experiment, an increase of urine output was observed in WT mice treated with the high gemfibrozil diet. The maximal change was about 6 times of the baseline level, which was similar to the 14-day treatment and remained similar through 60 days (*n*=5). Seven days after the diet replacement, the urine output quickly decreased and was almost normalized after another week (*n*=3). As expected, no modification of urine output was observed during the 2-month drug challenge in the *Ppara*-null mice (Fig. 6A).

Phenotypically, the body weight of WT mice and *Ppara*-null mice did not change over the 2-month treatment (Fig. 6B). Creatinine was increased and went back to baseline upon drug withdrawal (Fig. 6C and D), and no morphological changes were found in the kidney tissues (Fig. 6E-H). These data indicated kidney tolerance in the 2-month treatment with gemfibrozil.
Discussion

Clinically, reduction of blood pressure was reported to be associated with fibrate administrations (Borghi et al. 2004; Kim et al. 2003; Williams et al. 2005). But the underlying mechanism has not yet been revealed. In a rat model, gemfibrozil was found to relax contraction of the tail artery and abolish the contractile activities of uterus, duodenum, and bladder (Phelps and Peuler 2010). A recent study revealed relaxation of rat thoracic aorta by gemfibrozil when gemfibrozil, bezafibrate and fenofibric acid were compared. This effect was supposed to be associated with a reduction of intracellular calcium (Liu et al. 2012). The above suggested a reasonable pharmacodynamic basis for the reduction of blood pressure. In the present study, both gemfibrozil and fenofibrate were found to increase urine output in WT mice not in Ppara-null mice, suggesting a role for PPARα in this new pharmacodynamic effect which might contribute to reduction of blood pressure.

The differential effect on MS by fibrate agents has been reported. In hypertriglyceridemic patients with and without diabetes mellitus in two different studies, gemfibrozil treatment improved glucose metabolism and insulin action (n=12 and 11 respectively) (Avogaro et al. 1995; Shen et al. 1991). Recently, gemfibrozil not fenofibrate was found to down-regulated systemic glucose level and glycogen storage in the liver dependent on PPARα in a mouse model (Song et al. 2016). In a rat MS model, fenofibrate reduced the plasma lipid levels within a few days, but the fasting plasma insulin and insulin secretion response to glucose stimulation were also decreased (Liu et al. 2011). Additionally, no improvement of glucose tolerance and
metabolic markers was observed with fenofibrate in dyslipidemia subjects with impaired glucose tolerance or T2DM (Noguchi et al. 2011). In this study, the urine output by gemfibrozil treatment was almost 6 times baseline in the WT mice, an effect stronger than that of fenofibrate (6-fold vs 2-fold). Since high glucose level is often concurrent with hypertension in MS patients, the data in this study suggested stronger effectiveness of gemfibrozil for MS treatment compared with fenofibrate.

The therapeutic regimen of gemfibrozil in the clinic is about 1200 mg/day, with fenofibrate usually prescribed at 200-300 mg/day. In this study, ‘low’, ‘medium’ and ‘high’ gemfibrozil doses (0.375%, 0.75% and 1.5% respectively) and fenofibrate doses (0.075%, 0.15%, and 0.3%) were administered in pelleted diets for 14 days. Compared with the 69% increase of liver/body weight ratio noted with high-gemfibrozil treatment, the 120% increase in the high-fenofibrate group indicated a higher risk of hepatomegaly for fenofibrate. This difference should be taken into account for investigations in rodent models, because complicated biochemical events occur underlying hepatomegaly. However, it is not a big concern in the clinical situation because human livers are refractory to this pharmacodynamic effect of PPARα agonists.

A reversible rise of serum creatinine associated with fibrates was reported in the clinic. Increases of creatinine production and a reduction of prostaglandins have been suggested as the underlying mechanism (Sica 2009). In another a retrospective clinical report, 10 male patients showed a reversible deterioration in serum creatinine when treated with a fibrate agent (pre-treatment: 182+/-14 µM, medication: 247+/-16 µM).
µM, post-medication: 183+/−13 µM). The mechanism involved was related to renal hemodynamics (Lipscombe et al. 2001). In this study, kidney related toxicity was systemically assayed. With a 14-day treatment of either gemfibrozil or fenofibrate, no modification of serum biochemical markers or histopathology was observed. In the 2-month treatment with gemfibrozil, the toxic responses were similar as those of the 14-day treatment except that the increase of serum creatinine in WT mice was observed on day 60 which decreased to the levels found in Ppara-null mice.

Diuretics are recommended as first-line drugs in early treatment of patients with hypertension in the clinic. However, most of the diuretics lead to high risk of electrolyte disturbances and endocrine disorders (Chobanian et al. 2003). In this study, three ions were modified between the KO-C and KO-H after gemfibrozil treatment. Na⁺ and Cl⁻ changed very mildly except for K⁺ in KO mice. None of them were altered by fenofibrate in either mouse line (Supplementary Figure S3). Additionally, a change was noted in ADH and ALD among the groups of WT and Ppara-null mice in this study. AQP3, which plays a crucial role in glycerol metabolism dependent on PPARα (Patsouris et al. 2004), was not modified in kidney tissues in either WT or Ppara-null mice in this study. However, transcription of kidney transporters is only one of the steps in urine output regulation. So there might be mechanism related with transporters remains to be investigated. Considering the disagreement of the above biochemistry with pathology and phenotype, it was more reasonable to attribute these variations to metabolic regulation instead of diuresis.

In conclusion, a regulatory effect on urine output mediated by PPARα was
revealed with clinically-prescribed PPARα agonists and transgenic mice. Gemfibrozil increased urine output with less risk of hepatomegaly compared with fenofibrate. These data revealed a differential potential between gemfibrozil and fenofibrate for the treatment of hypertriglyceridemia patient with concurrent hypertension. However, the exact mechanism underlying increase of urine output by gemfibrozil remains to be investigated. Considering the species difference related with PPARα, these data in mice may not reflect results in humans.
Conflict of interest: The authors declare no conflicts of interest.

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References List


Figure 1. The urine output regulated in WT and KO mice treated with gemfibrozil or fenofibrate. A, The urine output in the WT mice treated with gemfibrozil for 14 days. B, The urine output in the KO mice treated with gemfibrozil for 14 days. C, Urine output of WT mice treated with fenofibrate for 14 days. D, Urine output of KO mice treated with fenofibrate. Data were expressed as mean ± SD (n=5; *p<0.05; **p<0.01; ***p<0.001; ns, not significant; WT, wild type; KO, Ppara-null).

Figure 2. Organs indices in WT and KO mice treated with gemfibrozil or fenofibrate. A, Organ indices in the WT mice treated with gemfibrozil. B, Organs indices in the WT mice treated with fenofibrate. C, Organs indices in KO mice treated with gemfibrozil. D, Organs indices in the KO mice treated with fenofibrate. Data were expressed as mean ± SD (n=5; *p<0.05; **p<0.01; ***p<0.001; ns, not significant; WT, wild type; KO, Ppara-null).

Figure 3. Biochemical and pathological responses of the WT and KO mice treated with gemfibrozil for 14 days. A, Serum levels of CRE. B, Serum levels of BUN. C-H: Representative H&E staining of kidneys from WT-C, WT-L, WT-M, WT-H, KO-C and KO-H mice. Mice were fed with control-, low-, medium-, high-gemfibrozil diets for 14 days. CRE and BUN were measured by clinical detection methodes. Data were expressed as mean ± SD (n=5; **p<0.01; ns, not significant; WT, wild type; KO, Ppara-null; CRE, creatinine; BUN, blood urine nitrogen).
Figure 4. Serum ion levels in mice treated with different dose of gemfibrozil. A, Serum Na⁺. B, Serum K⁺. C, Serum Ca²⁺. D, Serum Cl⁻. Mice were fed with control-, low-, medium- and high-gemfibrozil diets for 14 days. Data were expressed as mean ± SD (n=5; *p<0.05; ns, not significant; WT, wild type; KO, Ppara-null).

Figure 5. Hormone and mRNA levels related to urine production in mice treated with gemfibrozil. A, Serum ADH. B, Serum ALD. C, Aqp1, Aqp2, Aqp3 mRNAs. D, Nhe, Ncc, Nkcc1, Nkcc2 mRNAs. Serum hormones levels were measured by Elisa kit. Data were from kidney samples and the mRNA levels were measured by qPCR and normalized by 18S rRNA. Messenger RNA levels in WT-C mice were arbitrarily set as 1 and data were expressed as mean ± SD (n=5; *p<0.05; ***p<0.001; ns, not significant; WT, wild-type; KO, Ppara-null).

Figure 6. The phenotype, biochemical and pathological responses in the 2 month experiment of gemfibrozil. A, The urine output of mice treated with high-gemfibrozil for 2 months. B, Body weights of mice treated with high-gemfibrozil for 2 months. C, Serum creatinine (CRE) in the 2-month gemfibrozil experiment. D, Serum BUN in 2-month gemfibrozil experiment. E-H, Representative pictures of histological analysis of the kidney in mice treated with gemfibrozil. H&E staining of kidneys from WT-2m, KO-2m, WT-2m+14d, KO-2m+14d mice (WT, wild-type; KO, Ppara-null; d, days after dosing; c, days after clearance).
Figure 1

(A) Gemfibrozil

(B) Gemfibrozil

(C) Fenofibrate

(D) Fenofibrate

Urine output (mL/10 gm)

Time (day)

0 7 14

WT-C  WT-L  WT-M  WT-H

KO-C  KO-H

ns  ns  ns

ns  ns  ns

*  **  ***

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Figure 2

(A) Gemfibrozil

(B) Fenofibrate

(C) Gemfibrozil

(D) Fenofibrate
Figure 3
Figure 4

A. Na⁺ (mM)

B. K⁺ (mM)

C. Ca²⁺ (mM)

D. Cl⁻ (mM)
Figure 5