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Original Article

Gemfibrozil protect PC12 cells through modulation of Estradiol receptors against oxidative stress

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Abstract

Introduction: Neurodegenerative diseases are progressive disorders that could impair neuronal functions and structures. Oxidative stress and mitochondrial dysfunction are involved in the etiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and etc. Gemfibrozil is used as a therapeutic drug for hyperlipidemia. It has been shown that gemfibrozil is neuroprotective via modulation of mitochondrial biogenesis pathway under oxidative stress condition and in a sex-dependent manner.

Materials and Methods: In this study, neuronal-like PC12 cells with were pretreated with different concentrations of gemfibrozil and H_2O_2, concomitantly.

Results: In gemfibrozil pretreated groups, reduced level of caspase-3 and raised mitochondrial transcription factor A (TFAM) levels were detected. In contrast, adding fulvestrant, an Estradiol receptor antagonist, prevents the impact of gemfibrozil on oxidative stress condition, reducing its efficacy to protect the neurons against stress.

Conclusion: Our results indicated the involvement of estradiol receptors in gemfibrozil neuroprotective mechanism, in diminishing oxidative stress-induced damage via reducing caspase-3 and inducing the level of TFAM that plays a crucial role in the mitochondrial biogenesis.

Keywords:
Gemfibrozil;
Mitochondrial transcription factor A;
Fulvestrant;
Caspase-3;
H_2O_2

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Introduction

In many cases, the etiology of neurodegenerative diseases such as Alzheimer's, Huntington, Parkinson's disease and etc. (Rubinsztein, 2006), as well as aging (Lin and Beal, 2006) are directly related to oxidative stress and mitochondrial dysfunction. Oxidative stress is involved not only in pathogenesis and neuronal damage in the context of neurodegenerative diseases(Emerit et al., 2004), but also in a variety of disorders such as Diabetes(Maritim et al., 2003), Vascular disorders (Madamanchi et al., 2005), Myocardial infarction (Ramond et al., 2013) and Sickle cell anemia (Amer et al., 2006).

Mitochondrial biogenesis utilized as a protective mechanism in different conditions including hypoxia (Carre et al., 2010), oxidative injury (Rasbach and Schnellmann, 2007), focal and global cerebral ischemia (Chen et al., 2001; Chen et al., 2010)and

oxidative stress (Wei et al., 2001; Onyango et al., 2010), as well as metabolic disease (Knutti and Kralli, 2001; Finck and Kelly, 2006), brain stroke (Dong et al., 2007) and mitochondrial diseases (Wenz, 2009). Mitochondrial biogenesis is regulated by mitochondrial and nuclear genomes causing e mitochondrial proliferation and differentiation, which is altered during oxidative stress (Ostronoff et al., 1996; Fernandez-Silva et al., 2003). Mitochondrial transcription factor A (TFAM) is considered as a key player in initiating mitochondrial biogenesis (Miranda et al., 1999). According to some studies, induction of H_2O_2 produces reactive oxygen species (ROS) and ROS subsequently triggers the procaspase-3 cleavage and consequent release of cytochrome c from mitochondria, leading to apoptosis (Tang et al., 2005).

Gemfibrozil is a lipid lowering agent that belongs to a group of drugs known as fibrates(Xu et al., 2001a). It has been demonstrated that fibrates as peroxisome proliferator-activated receptor (PPAR)-α agonists, that protect against oxidative stress via anti-oxidant and anti-inflammatory mechanisms (Deplanque et al., 2003; Bordet et al., 2006; Xu et al., 2007). Also, it has been claimed that benzafibrate and fenofibrate also in the fibrate category could induce mitochondrial biogenesis in the skeletal muscle and liver (Nagai et al., 2002). Moreover, researches have proved that fibrates affect metabolism of steroid hormones and have direct estrogenic activity through binding to estrogen receptors as well (Xu et al., 2001a; Fan et al., 2004; Isidori et al., 2009). Estrogen receptor (ER) antagonist such as fulvestrant is mainly considered for treatment of hormone sensitive metastatic breast cancer(Osborne et al., 2004). Gemfibrozil has been able to significantly attenuate superoxide production resulting in inhibition of apoptosis (Calkin et al., 2006). Our recent studies have showed that gemfibrozil pretreatment resulted in a sexually-dimorphic Outcome (Mohagheghi et al., 2013a; Mohagheghi et al., 2013b). Gemfibrozil activated Nuclear respiratory factor 1 (NRF-1) and Mitochondrial transcription factor A (TFAM) in mitochondrial biogenesis signaling pathway and subsequently inhibited the caspase-dependent apoptosis, resulting in protection of female rats; while in male rats, provoked both caspase-dependent and caspase–independent apoptotic pathways and resulted in suppression of mitochondrial biogenesis signaling factors, meaning gemfibrozil acted reversibly and led to neurodegeneration (Mohagheghi et al., 2013a; Mohagheghi et al., 2013b).

On the basis of above results, we aimed to test the effect of three different dosages of (5, 10 and 20 μM) of gemfibrozil on TFAM and apoptotic factor (caspase-3) neuronal level in the differentiated rat pheochromocytoma cells being exposed to oxidative stress in two different time points (4 hours and 9 hours after induction), and then examined the extent of involvement of estrogen receptors when administrating fulvestrant (an antagonist of ER).

Materials and methods

Antibodies directed against caspase-3, TFAM and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Electrochemiluminescence (ECL) kit was purchased from Amersham Bioscience (Piscataway, NJ, USA). Fetal Bovine Serum (FBS) was provided from Gibco (Big Cabin, Oklahoma, USA), and polyvinylidene fluoride membrane was obtained from Chemicon Millipor (Temecula, CA, USA). All the other reagents were from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture and treatment conditions

Rat pheochromocytoma (PC12) cells were obtained from Pasteur Institute (Tehran, Iran) which were grown in Dulbecco's modified Eagle's medium (DMEM), enriched with 5% fetal bovine serum, 10% horse serum, and 1% antibiotic (penicillin-streptomycin), Cultures were maintained according to standard protocols at 37 °C in a 95% humidified incubator with 5% CO2 (Greene and Tischler, 1976). Growth medium was changed three times a week. The cells were differentiated by incubating with nerve growth factor (NGF; 50 ng/mL) for 6 days (Figure.1).

PC12 cells were treated with different concentrations (5, 10 and 20 μM) of gemfibrozil and 500 nM of fulvestrant. To induce oxidative stress 150 μMH_2O_2 was added to culture plates.

MTT cell viability assay

Cell viability was determined using a 3(4,5-dimethylthiazol-2yl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay(Mosmann, 1983) s.

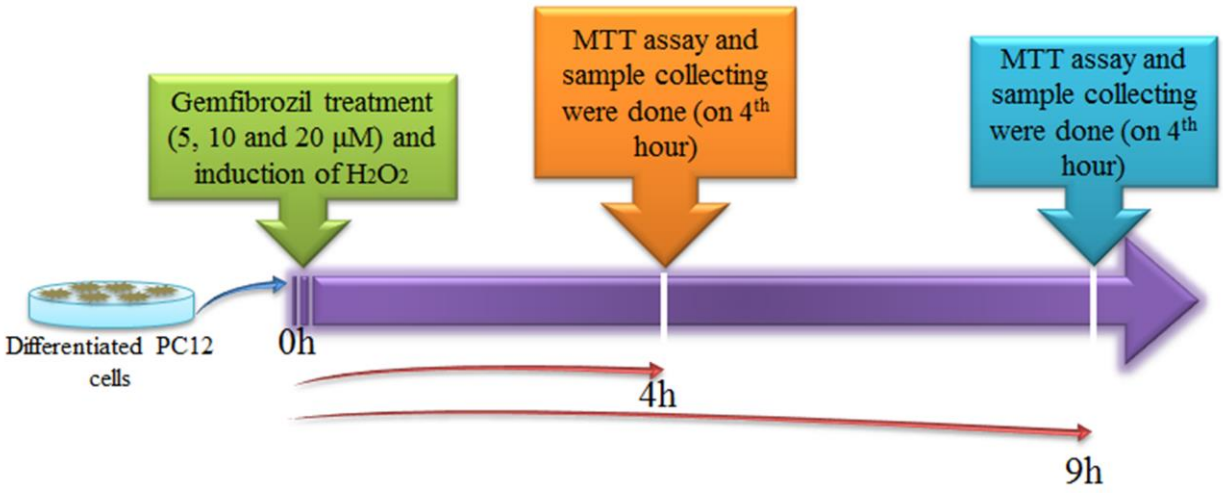

Fig.1. A schematic representation of experimental procedure. Neuronal like PC12 cells were pretreated with gemfibrozil (5, 10 and 20 µM of gemfibrozil) and H_2O_2. In 4 hours and 9 hours after induction of H_2O_2 and three doses of gemfibrozil in separate groups, the MTT assay was done and neurons were collected to measure caspase-3 and TFAM.

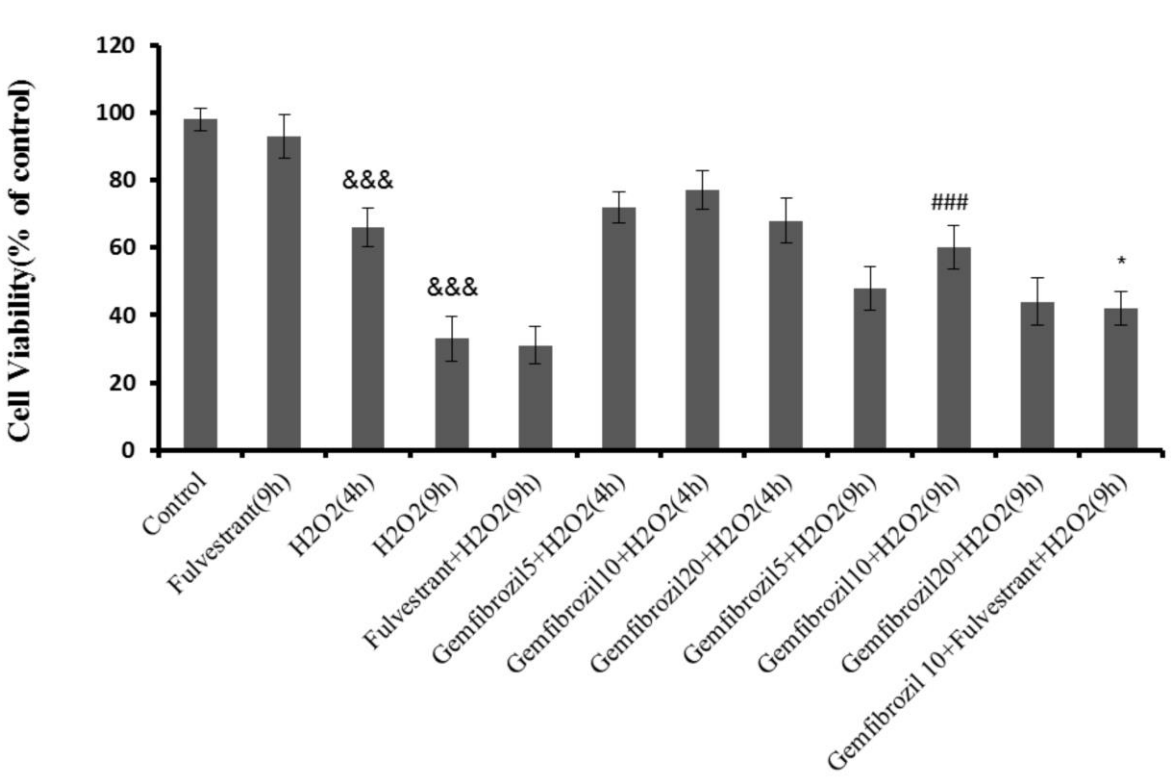

Fig.2. Effect of gemfibrozil and fulvestrant on cell viability against oxidative stress. The cell viability was determined by the MTT reduction assay and the surviving cell values were expressed as the percentage of control cells. Experiments were replicated 3 times independently. $^{\&\&\&}$ $p<0.001$ versus control. $^{\#\#\#}$ $p<0.001$ versus H_2O_2 (9 hours), * $p<0.05$ versus Gemfibrozil 10 µM + H_2O_2 (9 hours).

The dark blue formazan crystals formed in intact cells were solubilized in dimethyl sulfoxide, and the optical density (O.D.) of each well was measured with a spectrophotometer equipped with a 550 nm filter. Results were expressed as percentage of cell viability = (O.D. treated/O.D. control) × 100.

Western blot technique

For Western blot analysis, total proteins were electrophoresed in 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Then the membrane was incubated with specific antibodies. Immuno-reactivity was detected

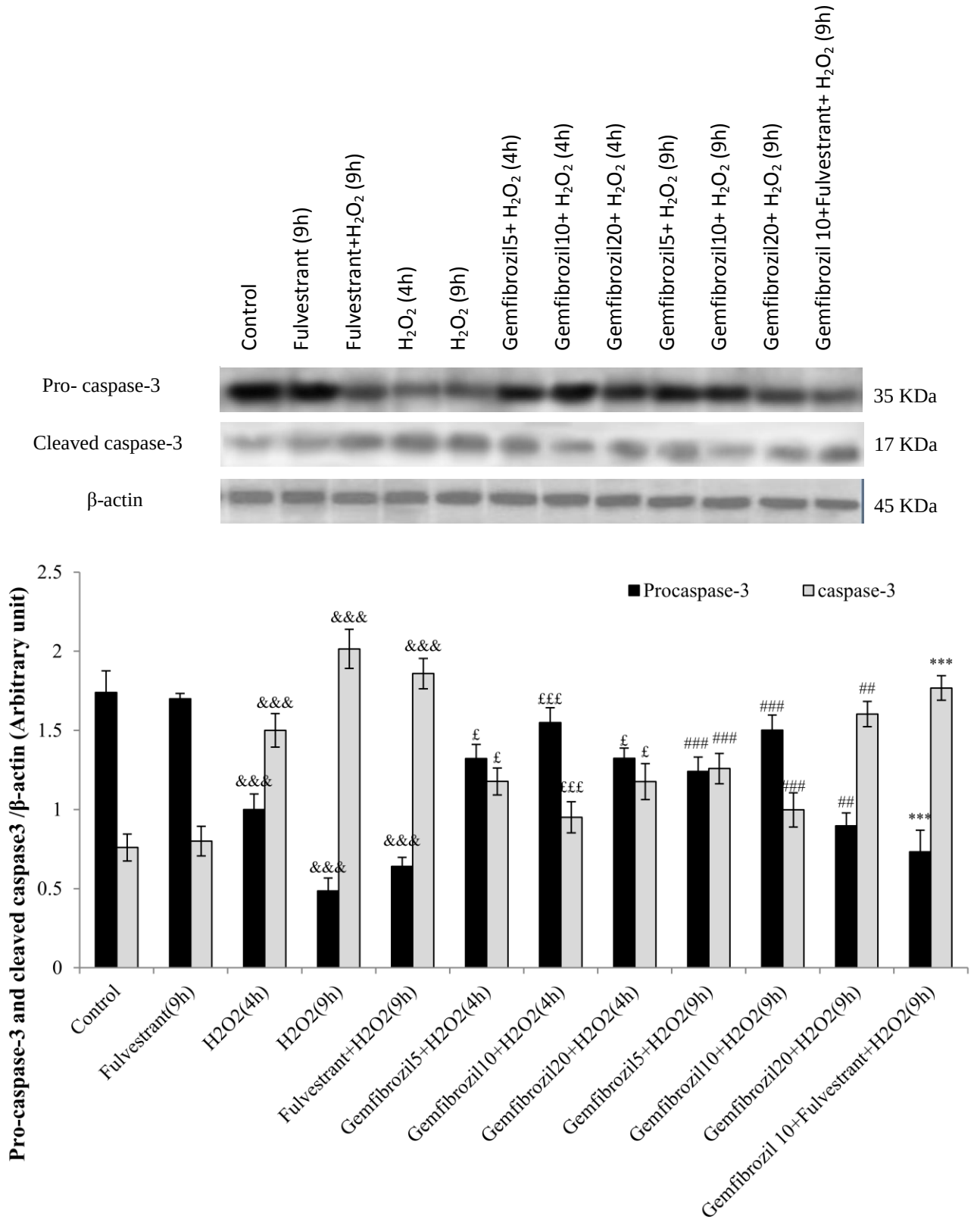

Fig.3. Effect of gemfibrozil and fulvestrant on pro- and cleaved capsase-3 level against oxidative stress. A) Cell lysate were prepared and subjected to western blotting with caspase-3. For normalization, membranes were stripped and reprobed with β-actin antibody. (One representative Western blot was shown) B) Quantitative detection of procaspase-3 and cleaved capsase-3 level in PC12 cells compared to β-actin. Each comparison was made with the same pro- or cleaved caspase-3. $^{\&\&\&}$ p<0.001 versus control in pro- and/or cleaved caspase-3, $^{£££}$ p<0.001 versus H_2O_2 (4 hours) in pro- and/or cleaved caspase-3, $^{\#\#\#}$ p<0.001 versus H_2O_2 (9 hours) in pro- and/or cleaved caspase-3, *** p<0.001 versus Gemfibrozil 10 μM + H_2O_2 (9 hours) in pro- and/or cleaved caspase-3.

by enhanced Electro Chemi Luminescence (ECL) reagent, followed by autoradiography. Band density was analyzed using Image.J. Concentration of protein was determined by Bradford protocol (Bradford, 1976).

Data analysis

All data are represented as the mean ± S.E.M (Standard Error Mean). Comparison between groups in cell viability and Western blot data was made using one-way analysis of variance (ANOVAs), and statistical significances were achieved with $P < 0.05$.

Results

Cell viability

MTT assay was used to measure the cell viability. In order to further examine protective potential of gemfibrozil against H_2O_2, cells treated with different concentrations of gemfibrozil (5μM, 10 μM and 20 μM) in different time (4 hours and 9 hours) periods. In addition, fulvestrant (ER antagonist) was added to gemfibrozil treated group (10 μM) after 9 hours, to evaluate probable role of ER receptors in gemfibrozil-induced protection.

As shown in the Fig. 2 gemfibrozil was more protective when added in a dose of 10 μM in comparison with two other doses (p value <0.001; versus "H_2O_2 (9 hours)"), and presence of fulvestrant reduced gemfibrozil-induced protection (p value <0.05; versus "Gemfibrozil (10 μM)+ H_2O_2 (9 hours)").

Expressions of Caspase-3 and TFAM detected by Western blotting

Pro-caspase-3 and cleaved caspase-3

In order to evaluate the effect of gemfibrozil and fulvestrant on apoptotic pathway, cells were treated with different concentration of gemfibrozil (5μM, 10 μM and 20 μM) in different times (4 hours and 9 hours) and the level of pro-caspase-3 and cleaved caspase-3 were detected by Western blotting technique. Then fulvestrant was added to the group receiving gemfibrozil (10 μM) in 9 hours.

As depicted in Fig. 3, gemfibrozil (10 μM) in 4 hours showed the most protective effect on PC12 cells against oxidative stress in cleaved caspase-3 and pro-caspase-3 (p value <0.001; versus "H_2O_2 (4 hours)" in both cleaved caspase-3 and pro-caspase-3). Gemfibrozil (10 μM) showed protective effect in 9 hours as well (p value <0.001; versus "H_2O_2 (9 hours)" in both cleaved caspase-3 and pro-caspase-3).

Fulvestrant inhibited the protection offered by gemfibrozil to the apoptotic pathway; as the expression of cleaved caspase-3 in the group of PC12 cells pretreated with "gemfibrozil (10μM) + fulvestrant in 9 hours" increased significantly;(p value<0.001; versus "gemfibrozil (10μM)+H_2O_2 (9 hours)" in both cleaved caspase-3 and pro-caspase-3).

TFAM

We also examined the effect of gemfibrozil and fulvestrant on TFAM, as a very important protein in mitochondrial biogenesis. PC12 cells were treated with different concentrations of gemfibrozil (5μM, 10 μM and 20 μM) in different times (4 hours and 9 hours) and the level of TFAM was detected. As represented in Fig. 4, gemfibrozil (10μM) reduced the damaging effect of oxidative stress on mitochondrial biogenesis (p value <0.001; versus "H_2O_2 (9 hours)") and this protective effect was seen 4 hs time span as well (p value <0.001; versus "H_2O_2 (4 hours)"). Fulvestrant (500nM) reduced gemfibrozil (10 μM) protection in 9 hours (p value <0.001; versus "gemfibrozil (10 μM) + H_2O_2 (9 hours)").

Discussion

In the current study, we examined the protective role of gemfibrozil in three doses (5, 10 and 20 μM) against oxidative stress. Cells were collected in two different time intervals (4 and 9hour) after exposure to H_2O_2. Our results revealed that the dose of 10 μM gemfibrozil was the most protective dose relevant to the three measured factors (MTT assay, Caspase-3 and TFAM) in PC12 cells. Fulvestrant, as an estradiol receptor antagonist inhibited the protective role of gemfibrozil (10 μM) affecting all parameters. During oxidative stress, the raised intracellular level of ROS leads to apoptosis and necrosis (Lennon et al., 1991). Moreover, necrosis commonly occurs in the last stage of apoptosis (Canu et al.,

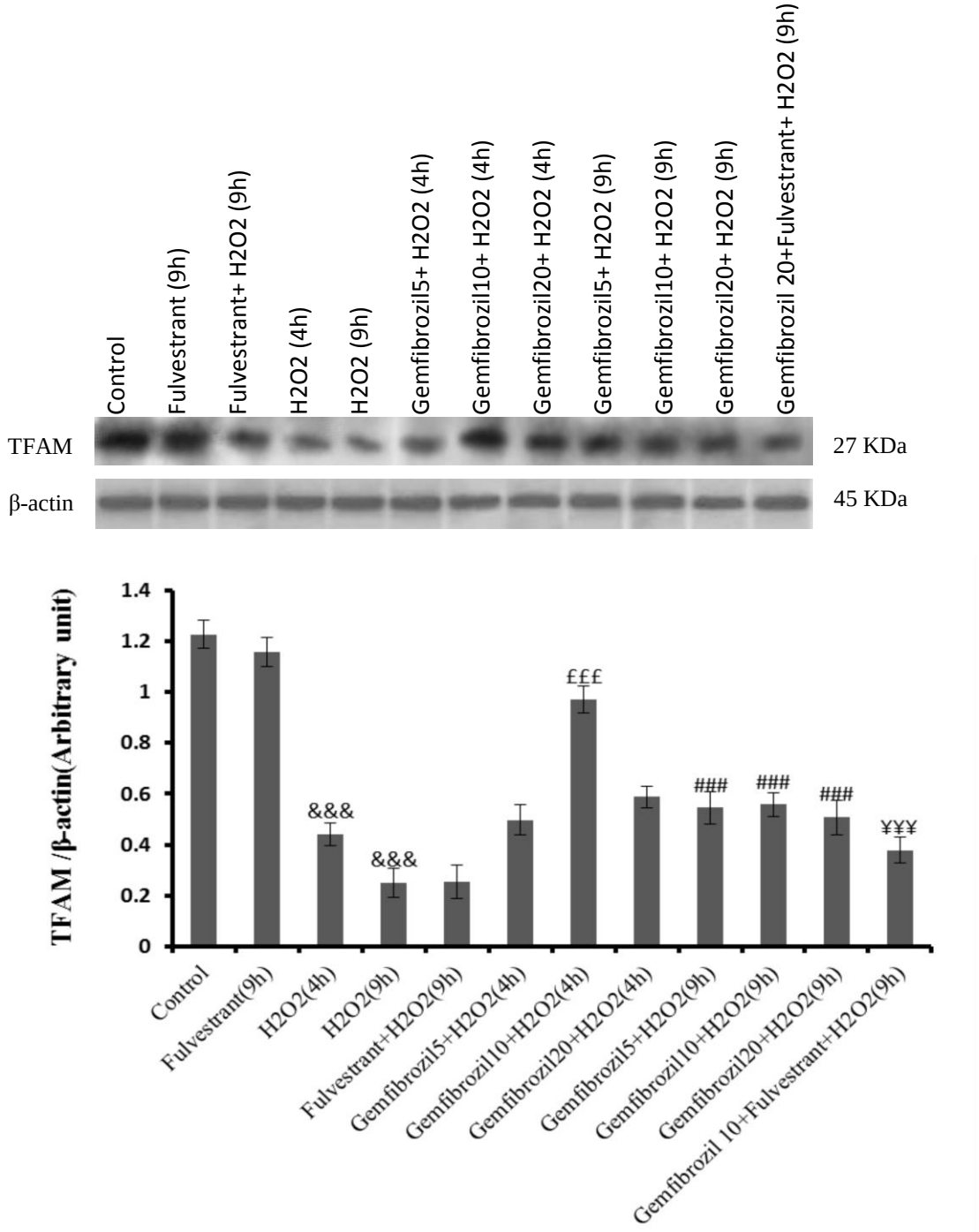

Fig.4. Effect of gemfibrozil and fulvestrant on TFAM (Mitochondrial transcription factor A) level against oxidative stress. A) Cell lysate were prepared and subjected to western blotting with TFAM. For normalization, membranes were stripped and reprobed with β-actin antibody. (One representative Western blot was shown) B) Quantitative detection of TFAM level in PC12 cells compared to β-actin. $^{\&\&\&}$ p<0.001 versus control, $^{£££}$p<0.001 versus H_2O_2 (4 hours), $^{\#\#\#}$ p<0.001 versus H_2O_2 (9 hours), $^{¥¥¥}$ p<0.001versus Gemfibrozil 10 μM + H_2O_2 (9 hours).

2014). Our Hoechst staining data revealed that apoptosis is more prevailing cell death mechanism among others (data not shown), therefore it can be assumed that gemfibrozil might reduce apoptosis and consequently increase cell viability. It is believed that oxidative stress is involved in the progression of many disorders such as neurodegenerative diseases, cancer and ischemia. During oxidative stress, leakage rate of ROS across mitochondrial membrane is elevated which triggers activation of some other harmful cellular signaling pathways (Seaver and Imlay, 2004). Many investigations have focused on eliminating cellular oxidative stress by detoxifying using pharmaceutical agents and natural compounds.

Reports have shown that a group of drugs called fibrates can ameliorate oxidative stress-induced toxicity in neurons (Mutez et al., 2009; Nakajima et al., 2010; Mohagheghi et al., 2013a). Gemfibrozil is one of these which helps in reducing hyperlipidemia (Hodges, 1976). Some recent studies have pointed to the neuroprotective potential of gemfibrozil (Corbett et al., 2012; Ghosh and Pahan, 2012; Khalaj et al., 2013). Furthermore, gemfibrozil attenuated the inflammatory response and oxidative stress by induction of antioxidant enzymes (Camara-Lemarroy et al., 2015). At some point in the past, there existed some controversial reports regarding the role of Gemfibrozil against ROS production.(Gust et al., 2013). Also, Scantena and colleagues showed that gemfibrozil increased ROS production in the phagocytic leucocytes (Scatena et al., 1997).

Estrogen receptors (ERs) are intracellular receptors which are activated by 17-β-Estradiol hormone. ER can regulate some cellular signaling pathways such as MAPK, PI3K/Akt, as well as G protein-coupled receptor (GPR30) (Prossnitz et al., 2007). PPARα and ERs are members of steroid hormone receptor superfamily (Boitier et al., 2003; Bain et al., 2007), and structurally have six functional domains (Wei et al., 2001). So, it is not far-fetched to assume that these receptors may have same ligands. Studies showed that there is a link between induction of fibrate drugs and estradiol releases (Gonzalez, 2002). It is reported that gemfibrozil has increased the metabolism of estrogen (Corton et al., 1997). Moreover, gemfibrozil has enhanced the release of estradiol in rat liver cells (Xu et al., 2001b).

Previous studies reported increased cell viability against stressful situations in various *in vitro* and *in vivo* experimental models (Jana and Pahan, 2012; Khalaj et al., 2013). Our study indicated that gemfibrozil has dose-dependent effects; as 10μM concentration of gemfibrozil increased cell viability in 9h after exposure to H_2O_2, while doses of 5 and 20μM of gemfibrozil were less protective against oxidative stress. So, we have U-shaped curves These curves show asymptotic response in high doses of drugs because of tachyphylaxis or receptor fatigue (Paterson and Day, 1979). Therefore, the dose of 10μM of gemfibrozil was considered as the most protective dose in this study. Besides, inhibition of ERs by fulvestrant decreased cell viability which shows gemfibrozil functions through ERs.

Our results revealed that gemfibrozil decreased cleaved caspase-3 levelin PC12 cells. In 4 and 9 time intervals after oxidative stress respectively, the level of capsase-3 was attenuated by gemfibrozil, while addition of fulvestrant reversed this protective effect. These data confirmed that gemfibrozil protective effect is modulated through ERs.

In our previous studies, we found a sex-dependent outcome of gemfibrozil pretreatment in rat cerebral global ischemic injury (Boitier et al., 2003; Bain et al., 2007). Therefore, herein we decided to further examine the role of ERs in the neurons receiving gemfibrozil against oxidative stress. Interestingly, gemfibrozil neuroprotective effects were reversed by fulvestrant, confirming involvement of ERs. This undetscores another crucial role of gemfibrozil in enhancing estradiol release (Xu et al., 2001b; Xu et al., 2001c; Isidori et al., 2009). Moreover, there are some clinical and experimental reports which have demonstrated a proven close correlation between ERs and gemfibrozil in other contexts (Goldenberg et al., 2003; Zenobio et al., 2014).

In the current study, we also evaluated the role of gemfibrozil on TFAM in the presence and absence of fulvestrant. Some of the main proteins in mitochondrial biogenesis are NRFs, PGC-1α and TFAM. Gemfibrozil has been shown to modulate the levels of mitochondrial biogenesis related factors (Miglio et al., 2012; Khalaj et al., 2013). Additionally, it is proven that ERs can be regulated by mitochondrial biogenesis factors (Villena et al., 2007). Our results showed that gemfibrozil enhanced TFAM level in a dose-dependent manner, and fulvestrant application attenuated the level of TFAM under oxidative stress condition. It has been proposed that gemfibrozil and other fibrates increased mitochondrial biogenesis factors by induction of PPARα (Sanoudou et al., 2010). Our time-course study indicated that the level of TFAM increased in 4h after oxidative stress and then decreased in 9h. While the cleaved capsase-3 level decreased in 4h and 9h after induction of H_2O_2. These data shows that gemfibrozil might have activated some protective pathways such as mitochondrial biogenesis, and consequently ameliorated activation of the caspase cascade. There are several reports indicating that activation of mitochondrial biogenesis triggered PPARα, and which subsequently induced protective pathways within the cells (Finck and Kelly, 2006; Finck and

Kelly, 2007). Also, induction of TFAM by gemfibrozil was inhibited by fulvestrant, which further confirmed that gemfibrozil acts via ERs. However, the precise mechanisms of gemfibrozil in activating mitochondrial biogenesis in the neurons await more researches.

Conclusion

The data obtained in our experiment demonstrated that gemfibrozil increased cell viability by inhibition of caspase-3 and induction of TFAM in the context of oxidative stress-induced neuronal damage. Dose-dependent study revealed that 10μM gemfibrozil was the most protective dose against oxidative stress. Administration of fulvestrant, an ERs antagonist, to the gemfibrozil pretreated cells enhanced caspase-3 and reduced TFAM level, and the data we presented here confirmed he ERs modulatory role against gemfibrozil protective effect in neuronal contexts.

Acknowledgment

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Conflict of Interest

The authors report no declarations of interest.

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