Research Article

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Effects of peroxisome proliferator activated receptor gamma (PPARγ) agonist on fasting model applied neuron cultures

Peroksizom Proliferatörle Aktive Edilmiş Reseptör Gama (PPARγ) Agonistinin Açlık Modeli Uygulanan Nöron Kültürleri Üzerindeki Etkileri

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Abstract

Objectives: Peroxisome proliferator activated receptor gamma (PPARγ) agonists used for the treatment of Diabetes Mellitus (DM), has important roles on the regulation of metabolism including ketogenesis in fasting and low glucose states. Recently PPARγ was proven to have antioxidant and anti-inflammatory effects on neuronal cells.
Methods: In the present study, effects of pioglitazone (PPARγ agonist) on cell survival, energy metabolism and mitochondrial functions were investigated in glucose deprived fasting model applied SH-SY5Y (ATCC/CRL 2266) cell lines. Before and after pioglitazone treatment; energy metabolites (glucose, lactate, ketone (βOHB), lactate dehydrogenase activity), mitochondrial citrate synthase activity and cell viability were investigated.
Results and Conclusions: PPARγ agonist addition to glucose deprived, ketone added neurons provided positive improvements in energy metabolites ($p<0.01$), mitochondrial functions ($p<0.001$) and survival rates ($p<0.01$). Changes in mitochondrial citrate synthase activity, lactate and LDH levels of neuronal cells treated with PPARγ agonist have not been previously shown. Our results suggest, pioglitazone as an effective alternative for the treatment of neurodegenerative diseases especially with the presence of ketone bodies. By clarifying the mechanisms of PPARγ agonists, a great contribution will be made to the treatment of neurodegenerative diseases.

Keywords: fasting; ketone bodies; neuron cultures; pioglitazone; PPARγ.

Özet

Genel Bilgiler: Diabetes Mellitus (DM) tedavisinde kullanılan Peroxisome Proliferatör ile aktive edilen Reseptör Gamma (PPARγ) agonistleri, açlık ve düşük glukoz durumlarında ketogenez dahil metabolizmanın düzenlenmesinde önemli rollere sahiptirler. Son zamanlarda PPARγ'nın nöron hücreleri üzerinde antioksidan, anti enflamatuvar etkilere sahip olduğu gösterilmiştir.
Amaç: Bu çalışmada; pioglitazonun (PPARγ agonisti) hücre canlılığı, enerji metabolizması ve mitokondriyal fonksiyonlar üzerindeki etkileri, SH-SY5Y (ATCC/CRL 2266) hücre

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hatlarına uygulanan glikozdan yoksun açlık modelinde araştırılmıştır.
Materyal ve Metot: Pioglitazone uygulanmasından önce ve sonra; enerji metabolitleri (glukoz, laktat, keton (βOHB), laktat dehidrogenaz aktivitesi), mitokondriyal sitrat sentaz aktivitesi ve canlılık oranları araştırıldı.
Bulgular ve Tartışma: Glukozu azaltılmış, keton ilave edilmiş (Açlık modeli) nöron kültürlerine PPARγ agonisti eklenmesi; metabolik parametrelerde ($p<0.01$), mitokondriyal fonksiyonlarda ($p<0.001$) ve canlılık oranlarında ($p<0.01$) pozitif yönde iyileşmelere neden oldu. PPARγ agonisti uygulanan nöronlarda mitokondriyal sitrat sentaz aktivitesi, laktat ve LDH seviyelerindeki değişiklikler daha önce gösterilmemiştir. Sonuçlarımız, pioglitazonun özellikle keton cisimciklerinin varlığında nörodejeneratif hastalıkların tedavisinde etkili bir alternatif olduğunu düşündürmektedir. PPARγ agonistlerinin etki mekanizmalarının açıklığa kavuşturulması, nörodejeneratif hastalıkların tedavisine büyük katkı sağlayacaktır.

Anahtar Kelimeler: Açlık; Keton Cisimcikleri; Nöron Kültürleri; Pioglitazone; PPARγ.

Introduction

In recent years, effects of calorie restriction (CR) and prolonged fasting on brain functions have been studied in experimental and clinical studies. In both cases, since there is a glucose restriction, brain uses ketone bodies as an alternative energy source. It is known that ketogenic diet is strongly effective in epileptic patients who are resistant to anti-epileptic drugs [1]. Ketogenic diet primarily consists of high-fats, moderate-proteins, and low-carbohydrates which leads the body to synthesize ketones as a result of low glucose in blood. Our group is working on the effects of the prolonged fasting model, an alternative to ketogenic diet, and ketone bodies in humans and neuronal cells.

The present study is inspired by the newly discovered positive effects of peroxisome proliferator-activated receptor (PPAR) gamma agonists (PPARγ), used for the treatment of Diabetes Mellitus (DM), on neuron and mitochondrial damage [2, 3]. Ketogenic diet is shown to activate PPARγ by many mechanisms [2, 4]. In prolonged fasting ketone bodies, secreted in high concentrations, can also activate PPARγ pathways.

Peroxisome proliferator-activated receptors (PPARs) are proteins belong to superfamily of nuclear hormone factors, that are nuclear receptors and ligand dependent transcription factors. Structurally, PPARs are similar to steroid or thyroid hormone receptors and are stimulated in response to small lipophilic ligands. It is shown that they have critical role on the regulation of mitochondrial biogenesis, inflammation, anti-oxidant defense and pathways related to carbohydrate and lipid metabolisms [5]. PPARs have three isotypes (PPARα, PPARδ and PPARγ) and play an essential role in the energy metabolism; however, they differ in the spectrum of their activity and tissue distribution; PPARγ regulates energy storage, whereas PPARα is expressed predominantly in the liver, muscle, heart and bone and PPARδ is ubiquitously expressed in whole body regulating the energy expenditure [3].

Thiazolidinediones (TZDs) are widely studied PPARγ ligands. Acting as sensors of hormones, vitamins, endogenous metabolites, and xenobiotic compounds, PPARγs are nuclear receptors which control the expression of a very large number of genes. PPARγ has been known to regulate adipocyte differentiation, fatty acid storage and glucose metabolism, which makes it a target of antidiabetic drugs [6–8]. PPARγ agonist improves insulin resistance by opposing the effect of TNF-α in adipocytes [9]. Moreover, PPARγ agonists are shown to have effects in Parkinson, Alzheimer disease and brain injury. They are thought to act on microglial cells inhibiting their activation. Recently, PPARs have been shown to modulate inflammation by inhibiting the production of proinflammatory molecules by glial cells. *In vivo* oral administration of the PPARγ agonist pioglitazone reduced glial activation and the accumulation of Aβ-positive plaques in the hippocampus and cortex [2, 3].

All these information about the effects of PPARγ on neurodegenerative damage made us think that, PPARγ agonist pioglitazone may also be effective on neuronal mitochondrial functions together with ketone bodies. Our aim was to investigate if pioglitazone can be an alternative in the treatment of neuron damage together with ketone bodies. In our study, SH-SY5Y (human neuroblastoma-ATCC/CRL 2266) cell cultures were performed and glucose deprived fasting model was applied to these neurons. In physiological conditions, fasting is accompanied by ketone bodies so neurons were treated by beta-hydroxybutyrate and further by pioglitazone to investigate the effects on the energy metabolites, mitochondrial functions and viability of the cells.

Materials and methods

Cell cultures

During our study, SH-SY5Y (human neuroblastoma-ATCC/CRL 2266) cell line was purchased from ATCC. Frozen cells were thawed as indicated in the ATCC booklet. Cells were seeded at 2×10^5 cells/cm^2

density into in 25 cm^2 flasks and grown in media containing Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient mixture (DMEM/F-12) (1:1) containing L-glutamine and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 + 95% O_2 air atmosphere. When cells were 80–90% confluent, they were detached with trypsin and taken into new flasks/plates in order to perform the experiments. Cells were passaged to 75 cm^2 flasks when expected confluency was reached and the total media volume was made up to 10 mL before incubating at aforementioned conditions. Growth medium was replaced in every two days and doubling time studies showed up to be approximately 29 h. Experiments were performed in 75 cm^2 flasks and early passages of cells (P4-8 after purchase) were used.

Experimental groups and cell treatment conditions

Six groups were planned in the study (Table 1): control group (N), cells were incubated with normal medium which contained 300 g/dL glucose (DMEM/F12 + 10% FBS) (DMEM/F12, Gibco-11330057; FBS, Gibco-26140079). Glucose deprived fasting model group (F): Earl's balanced salt solution (EBSS, Sigma-E2888) + 10% FBS medium was used in this group (containing 100 g/dL glucose). Two sub-groups were formed by adding 5 mM ketone (Beta-hydroxy butyrate-βOHB, Sigma Aldrich-H9408) to the aforementioned two groups in order to simulate the physiological fasting conditions (FK) and ketone control group (NK). And finally for pioglitazone (Sigma Aldrich, E6910), (PPARγ agonist) added media, the abbreviation P is added to be NKP and FKP (Table 1). These latter groups were designed specifically to observe the behaviors of cells when only ketone presence and both ketone and pioglitazone presence in the media.

Betahydroxy Butyrate (βOHB, Sigma Aldrich-H9408) medium: βOHB was used in the experiments since it is the most potent ketone body. Calculations were made from βOHB (126.09 g/mol) to have a total of 5 mM in a 10 mL medium. Briefly, 0.630 mg βOHB weighed and dissolved in 50 mL sterile PBS, filtered with a 0.22 μm syringe to prepare the sterile stock solution. All the ketone-added media were prepared to have a final concentration of 5 mM βOHB.

Preparation of PPARγ Agonist medium; For a final concentration of 5 μM pioglitazone (Sigma Aldrich, E6910), 5 μL pioglitazone stock solution was added to 5 mL of the experiment media [10, 11].

Table 1: Experimental groups and media content.

	Medium	FBS	βOHB	PPARγ agonist	Glucose content, mg/dL
N	DMEM/F12	10%	0	0	300
NK	DMEM/F12	10%	5 mM	0	300
NKP	DMEM/F12	10%	5 mM	5 μM	300
F	EBSS	10%	0	0	100
FK	EBSS	10%	5 mM	0	100
FKP	EBSS	10%	5 mM	5 μM	100

Normal (N) and fasting (F) media were used as control groups, they consist neither ketone (βOHB) nor pioglitozone (PPARγ agonist). NK and FK groups were used as control groups of pioglitazone added groups (NKP and FKP). DMEM/F-12, Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient mixture; EBSS, Earl's balanced salt solution; FBS, fetal bovine serum.

Experiments

When cells were 80% confluent, normal media were withdrawn, flasks were washed with 2 mL of sterile phosphate buffered saline (PBS) and experiment media were added. 1 mL of the unused media were allotted for the measurements of energy metabolites (glucose, lactate, βOHB and Lactate dehydrogenase – LDH) before the experiments (initial values, *i*). Experiments were started the day before, and ended after 16-h incubation next morning. Experiment media were removed, 1 mL of them were allotted for the metabolic measurements again (end values, *e*). Cells were removed with Trypsin/EDTA and the viability rates were controlled with trypan blue. Then cells were homogenized for citrate synthase activity. In addition, microscopic pictures were taken (initial and after the experiment) for morphological examinations. Experiments were repeated 7 times under the same conditions.

Cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay is a cytoxicity experiment that is used to demonstrate the viability of cells numerically under certain conditions. MTT dye is metabolized by living cells and gives color at 595 nm wavelength, white color formation is not observed in dead cells. Color formation intensity is directly proportional to the cell viability. MTT Kit (Cat. Number 11465007001-Roche-Germany) was used in the experiments.

Analysis of metabolic parameters

Abbot precision-Xtra POCT device (California, USA) was used to measure ketone (βOHB) concentrations of the media before and after the experiments. 10 μL of each medium was introduced to ketone probe and measurements were performed. Glucose, lactate and LDH levels in the culture media were measured in ADVIA 1800 (Siemens-Germany) biochemistry auto-analyzer. Analytic range of the glucose measurement method was 1–700 mg/dL. For lactate measurements, the analytic range of the method was 2–50 mg/L. Lactate dehydrogenase activity was assessed by measuring the decrease in the optical density resulting from the oxidation of NADH at 340 nm using pyruvate as a substrate. Analytic range of the LDH activity measurement was 5–750 U/L.

Citrate synthase activity measurements

Citrate synthase is the first enzyme of the tricyclic acid cycle in the mitochondria matrix. The activity of citrate synthase is frequently used as a biochemical marker for the mitochondrial content and functions of a cellular homogenate. Citrate synthase activity measurement kit (Abcam-USA) was used for the enzyme activities in accordance with the experimental plan. Briefly, according to the protein concentrations, homogenates were diluted with the buffer solution to the expected protein concentration. Samples were added to a 96-well plate in a volume of 100 µL and incubated for 3 h at room temperature. Absorbances were measured in the ELISA reader and color changes were recorded at 412 nm for 30 min. Activity graphs were drawn and the time for Vmax of the enzymes was calculated as minutes for each group.

Data analysis

Our data are presented as mean ± standard deviation (SD) from seven independent experiments. Our results were evaluated statistically by one-way ANOVA, followed by Tukey's multiple-comparison test. Differences with a probability (p) less than 0.05 were considered statistically significant.

Results

Optimization studies

Metabolic parameters and cell viability with the vehicle

Final concentration of 5 µM pioglitazone was added to the neurons that were incubated with normal (N) and fasting (F) medium in order to see the effects of piaglitazone on metabolic parameters and viability of the cells and no statistically significant effect was detected on the cells (Table 2). According to these data, experiments were carried out without NP and FP groups.

Fasting model optimization studies

To determine the incubation period for glucose deprivation model, 10^5 cells were planted in 12-well flasks, each with an area of 4 cm^2, in the 6th passage and when the density was 80% normal growth media were removed and experiment media were added in three different groups: normal (N), fasting (F) and no glucose (G0). These media were added in a total volume of 2 mL according to the flask plan and cells were incubated in the aforementioned media for 4, 8, 12, 16, and 20 h. Media were collected from the wells at the beginning and at the end of each incubation, and the amount of glucose, lactate, LDH were analyzed (data not shown) together with cell viability (Figure 1). This experiment was repeated three times and 16 h of incubation period was decided by comparing all the results obtained. This decision was based on the LDH levels of the media, viability of the cells and the simulation of fasting for more than 12 h.

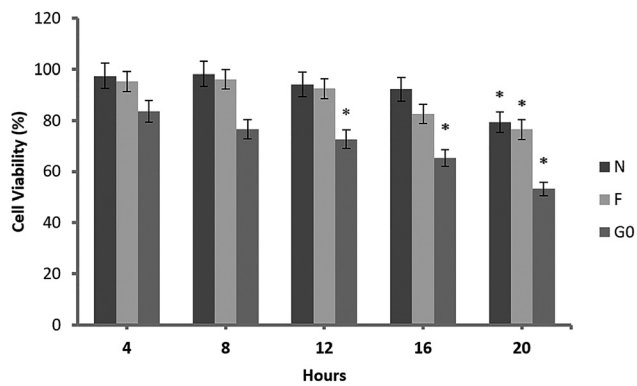

Figure 1: Cell viabilities in the normal and glucose deprived model optimization studies (n=3). N, cells were incubated with DMEM/F12 + 10% FBS. **F**, cells were incubated with EBSS + 10% FBS. **G0** (DMEM0 + 10% FBS, glucose-free medium), *p<0.01 when compared to the first 4 h of the same group.

Table 2: Comparison of metabolic parameters in optimization studies.

n=3	$Lactate_i$, mg/dL	$Lactate_e$, mg/dL	$Glucose_i$, mg/dL	$Glucose_e$, mg/dL	LDH_i, U/L	LDH_e, U/L	$Viability_e$, %
N	14.6 ± 1.3	22.1 ± 0.92	294 ± 13	249 ± 11	18.9 ± 1.7	27.9 ± 2.6	95
NP	13.9 ± 0.94	20.7 ± 0.83	299 ± 17	256 ± 16	17.4 ± 2.8	29.4 ± 1.7	98
F	14.7 ± 1.1	36.1 ± 1.8	98 ± 13	72 ± 12	16.3 ± 0.73	32.5 ± 2.9	82
FP	14.2 ± 0.83	37.6 ± 1.3	96 ± 9	78 ± 13	18.2 ± 1.7	27.9 ± 1.6	80

Values are shown with "*i*" before the experiment and with "*e*" after the experiment. N, cells were incubated with DMEM/F12 + 10% FBS. NP, cells were incubated with DMEM/F12 + 10% FBS + 5 µM PPARγ agonist (pioglitazone). F, cells were incubated with EBSS + 10% FBS. FP, cells were incubated with EBSS + 10% FBS + 5 µM PPARγ agonist (pioglitazone). Incubation period was 16 h.

Experimental energy metabolites

All cells consumed glucose during the 16 h experimental conditions, but cells incubated with ketone added media also used βOHB simultaneously, and their glucose utilization decreased compared to other cells (Table 3). In addition, lactate formation was reduced in ketone-added cells compared to other media together with LDH levels. Moreover, pioglitazone seemed to help the cells use their mitochondria to get energy rather than the anaerobic glycolysis. Lactate dehydrogenase enzyme activity measurements are one of the indicators of cell damage [12] and LDH levels of cells that were incubated with ketone and pioaglitazone added media were lower.

Cell viability

Cellular viability was assessed by the MTT assay. Cell survival was expressed as percentage neuroprotection compared with normal group at 100% (Figure 2). Viability rates were not different in neurons incubated in the normal media with or without ketone and PPARγ agonist. However, viabilities of ketone and PPARγ agonist treated fasting group cells were higher (p<0.01, Figure 2).

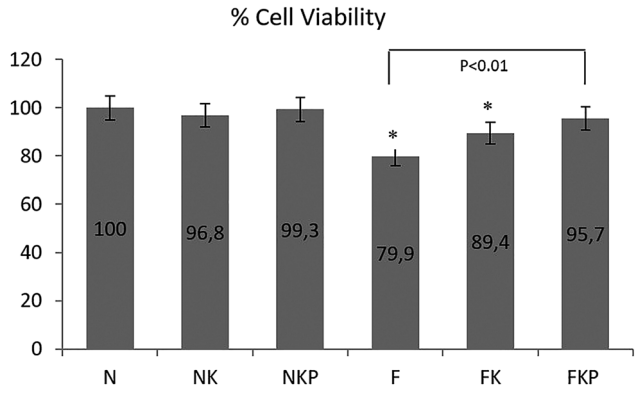

Figure 2: Cell viability is assessed with MTT assay. Data are expressed as mean ± SD (n=7). N, (normal) control group (DMEM F12 + 10% FBS-300 mg/dL glucose containing medium), NK, ketone added to normal medium (DMEM F12 + 10% FBS + 5 mM ßOHB), NKP, PPARγ agonist added to NK medium (DMEM F12 + 10% FBS + 5 mM ßOHB + 5 μM PPARγ agonist) F, glucose deprived media (EBSS + 10% FBS, 100 mg/dL glucose containing medium), FK, ketone added glucose deprived media (EBSS + 10% FBS, +5 mM ßOHB), FKP (PPARγ agonist added to FK medium (EBSS + 10% FBS, +5 mM ßOHB + 5 μM PPARγ agonist). *p<0.01, when compared to normal (N).

Citrate synthase activity

In our experiments, citrate synthase activity was measured in cells after 16 h of incubation with the experiment media. From the activity graphs, the time when the enzyme reaches the highest speed (Vmax) was calculated in minutes and is given in Figure 3. Cells incubated with normal medium reached the Vmax value fastest. It was observed that enzyme was working slowly as medium glucose content was decreased (Figure 3). In cells with the normal media, enzyme activity did not change significantly although mean velocity was slightly increased in cells containing both ketone and PPARγ agonist together (Figure 3). Enzyme velocity was found to be statistically increased in media containing ketone and further PPARγ agonist (Figure 3) in the fasting group. Additionally, enzyme activity observed to be faster in FK and FKP group than the F group which suggests in the deprivation of glucose, cells use ketone efficiently and may restore mitochondrial energy pathways in the presence of PPARγ.

Table 3: Energy metabolites before and after the experiments in the cell media.

	Glucose$_i$, mg/dL	Glucose$_e$, mg/dL	Lactate$_i$, mg/dL	Lactate$_e$, mg/dL	ßOHB$_i$, mmol/L	ßOHB$_e$, mmol/L	LDH$_i$, U/L	LDH$_e$, U/L
N	305 ± 16	231 ± 14†	14.3 ± 0.9	21.6 ± 1.6†	0	0	17 ± 2.1	23 ± 2.7†
NK	301 ± 18	270 ± 28*†	13.1 ± 1.2	22.5 ± 1.2$^{\dagger\#}$	5.3 ± 0.3	3.9 ± 0.5†	16 ± 3.6	24 ± 2.1*†
NKP	298 ± 12	282 ± 11*†	12.9 ± 1.5	19.5 ± 1.1†	5.1 ± 0.7	3.2 ± 0.7†	17 ± 4.1	20 ± 2.6*$^{\dagger£}$
F	99 ± 13	63 ± 9*†	14.2 ± 3.1	30.6 ± 2.8*†	0	0	18 ± 1.4	32 ± 0.9*†
FK	102 ± 9	77 ± 6*$^{\dagger\#}$	13.9 ± 2.2	24.3 ± 1.7*$^{\dagger\#}$	4.8 ± 0.8	3.1 ± 0.9†	17 ± 2.1	23 ± 1.7*†#
FKP	99.1 ± 11	79 ± 7$^{\dagger\#£}$	14.9 ± 2.1	19.3 ± 1.3*$^{\dagger\#£}$	5.1 ± 0.9	2.1 ± 0,7$^{\dagger£}$	18 ± 2.3	19 ± 1.5*$^{\dagger£}$

Values are shown with "*i*" before the experiment and with "*e*" after the experiment. Data are expressed as mean ± SD (n=7). N, (Normal) control group. NK, ketone added to normal medium. NKP, PPARγ agonist added to NK medium. F, glucose deprived media. FK, ketone added to glucose deprived media. FKP, PPARγ agonist added to FK medium. *p<0.01 when compared to N, $^{\#}$p<0.01 when compared to the same group without βOHB. †p<0.01 when compared to the values before the experiment at initial (*i*), $^{£}$p<0.01 when compared to the same group without pioglitazone.

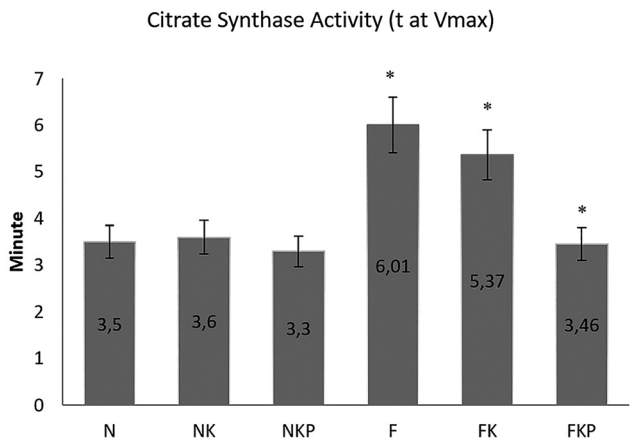

Figure 3: Citrate synthase activity was assessed after 16 h incubation with the experimental media. The time, when the enzyme reaches at the highest speed (Vmax) is calculated in minutes and given in the graphic (*p<0.01, when compared to normal). Data are expressed as mean ± SD (n=7). N, (normal) control group (DMEM F12 + 10% FBS-300 mg/dL glucose containing medium), NK, ketone added to normal medium (DMEM F12 + 10% FBS + 5 mM ßOHB), NKP, PPARγ agonist added to NK medium (DMEM F12 + 10% FBS + 5 mM ßOHB + 5 μM PPARγ agonist) F, glucose deprived media (EBSS + 10% FBS, 100 mg/dL glucose containing medium), FK, ketone added glucose deprived media (EBSS + 10% FBS, +5 mM ßOHB), FKP (PPARγ agonist added to FK medium (EBSS + 10% FBS, +5 mM ßOHB + 5 μM PPARγ agonist).

Discussion

In the present study, effects of peroxisome proliferator activated receptor gamma (PPARγ) agonist (pioglitazone) on glucose deprived, ketone added (fasting model) neuron cultures in terms of neuron viability, energy metabolism and mitochondrial functions were investigated in order to evaluate alternative treatment options in neurodegenerative diseases.

SH-SY5Y (human neuroblastoma-ATCC/CRL 2266) cell line was used in the study. SH-SY5Y is a human derived cell line used *in vitro* models of neuronal function and differentiation. The original cell line was isolated from a bone marrow biopsy taken from a four-year-old female with neuroblastoma. Normal nutrition and fasting models were applied to the 80% confluent cultured neurons by changing the glucose concentrations of the media [13]. Experiments were carried out by adding ketone and pioglitazone separately or together to the media.

When pioglitazone is added to the normal (N) and fasting (F) media alone it does not cause any statistically significant difference in metabolic parameters and cell viability in neurons (Table 2). However, when we simulate physiologic fasting conditions with ketone addition to the media (F and FK vs. FKP), metabolic parameters and cell survival rates were all changed in a positive manner (Table 3 and Figure 2). In the fasting group when pioglitazone was added to ketone containing medium (FKP), lactate and LDH levels were significantly lower than the F and FK group. Additionally neurons used ketones more efficiently in the presence of PPARγ agonist (Table 3). These findings made us think that, when pioglitazone added to the media, neurons might have used their mitochondria more efficiently (Figure 3). So we have conducted an other study with citrate synthase activity of the cells. Since citrate synthase is the first enzyme of Krebs cycle which takes place only in the mitochondrial matrix, activity measurements of citrate synthase in a cell homogenate, is accepted to show the mitochondrial capacity of a cellular community [14]. Growing evidence suggests the involvement of mitochondrial dysfunction in the pathogenesis of many major neurodegenerative and neuroinflammatory disorders. This dysfunction is associated with defects in mitophagy, bioenergetics, mitochondrial functions such as electron transport chain activity reductions and increased mitochondrial-generated oxidative stress [2]. Therefore, pharmacological approaches to enhance mitochondrial function, reduce reactive oxygen species (ROS) or enhance antioxidant defence are essential for the new therapeutic approaches to neurodegenerative and neuroinflammatory diseases.

It is shown that, knockout of PPARγ is embryonically lethal [15, 16]. Moreover, PPARγ activation is associated with increased lipid and carbohydrate metabolism together with adipocyte differentiation and reduction of plasma glucose concentration [17]. PPARγ agonists are currently in clinical use for the management of type 2 diabetes and pioglitazone, one of the most frequently used PPARγ agonists, was also been proven to have anti-inflammatory effects by PPARγ activation and regulating myelin gene expression in brain cells [18]. It is known that, PPARγ is expressed in different parts of brain. High levels of expression of PPARγ have been found in discrete areas of the central nervous system (CNS), including the dopaminergic cells in the basal ganglia [19, 20]. PPARγ, is also thought to regulate inflammatory response by suppressing the expression of more than one proinflammatory genes [21]. Moreover it is shown that inhibiting mammalian target of rapamycin (mTOR) pathway with PPARγ agonists had an anti-inflammatory effect on status epilepticus models in rats [22]. mTOR pathway inhibition is one of the major mechanisms of fasting in humans leading to ketogenesis [23].

In the present study, we have showed positive effects of PPARγ agonist on neuron survival, energy metabolism and mitochondrial functions which were enhanced with ketone body (βOHB) presence in the media. Changes in

mitochondrial functions and survival rates had been studied with PPARγ agonists, pioglitazone and rosiglitazone on differentiated SH-SY5Y neuroblastoma cells before [24]. Miglio et al. [24] showed PPARγ agonists promoted biogenesis of mitochondria in glucose deprived neurons while did not significantly change mitochondrial membrane potential. However, to our knowledge this is the first study showing positive effects of pioglitazone on neurons can be synergistically increased by ketones.

Ketone body supply and metabolism is essential in the brain. Ketolysis accounts for 70% of the energy supply during prolonged fasting (the majority is from βOHB), and the rest, comes from glucose derived from different sources: glycerol, amino acids, lactate and pyruvate catabolism [23]. Apart from their roles in the fasting metabolism, more recently, ketone bodies have been shown to have direct signaling capabilities [1]. There are multiple mechanisms through which ketone bodies have been thought to affect cellular signaling by modulating the activity of histone deacetylases (HDACs) [2, 23]. Ketone bodies, particularly βOHB, also shown to have anti-inflammatory effects by reducing inflammatory cytokine production, which may further limit neuronal cell loss and facilitate tissue regeneration following injury. With these aforementioned findings and in the light of our results we can suggest that PPARγ agonists and ketones may act synergistically in neuron protection. It has been shown that PPARγ agonist (pioglitazone) treated rats had increased ß-oxidation [25] which can enhance ketogenesis explaining the synergistic effects of these two on neuron protection.

There are certain limitations of this study. The present study is performed in secondary cell cultures with neuroblastoma cells which can have some differences in the regulation of metabolic pathways hence our results must be confirmed by primary neuron culture studies. Second limitation is, we have used βOHB as key ketone body in the treatments and did not test acetoacetate which is another ketone body synthesized by the liver. Another limitation is although it is shown that pioglitazone as the main molecule affects the metabolism, its metabolite hydroxypioglitazone is shown to be effective *in vivo* [25]. We have not tested its effects in the experiments. Finally mitochondrial functions were measured with metabolic parameters and citrate synthase activity in the present study that need to be confirmed with other investigations such as flowcytometric membrane potential analysis.

However, PPARγ agonist addition to glucose deprived and ketone added (fasting model applied) neurons caused positive improvements with metabolic parameters, mitochondrial functions, and survival rates. Changes in mitochondrial citrate synthase activity, lactate and LDH levels of neuronal cells treated with PPARγ and βOHB have not been previously pointed out in the literature. Based on all these findings; we can conclude that improvements in neuron damage were recorded after pioglitazone (PPARγ agonist) treatment especially in the presence of ketones. We suggest that the PPARγ agonists in fasting states show significant promise as therapeutic agents in neurological diseases and repositioning of pioglitazone may be a new approach for the treatment of neurodegenerative diseases. However, the role of pioglitazone in the treatment of neurodegenerative diseases and the integration with ketones should be further investigated by functional genetic pathway analysis and clinical studies.

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