Determination of neuroprotective effects of medium chain fatty acids and their derivatives on mutant huntingtin aggregates, oxidative stress and ATP levels in HD150Q cell line model of Huntington’s disease

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Huntington's disease (HD) is a rare autosomal dominant neurodegenerative disorder, occurring with an incidence of 1 in 10,000 individuals and has no cure. The etiology of HD involves an abnormal expansion of CAG repeats in exon 1 of the huntingtin (Htt) gene that translates into expanded polyglutamine (polyQ) tract in mutant huntingtin protein (mHTT). PolyQ expansion alters Htt structure resulting in abnormal protein-protein interactions, aggregation, mitochondrial dysfunction, oxidative and endoplasmic reticulum stress, inflammation and altered gene expression leading to neuronal cell death. HD symptoms involve chorea, dementia, behavioural and psychological problems and currently there is no cure highlighting the need for novel therapeutic interventions. Several fatty acids have been reported to have protective effects in neurological disorders including Alzheimer’s disease, Parkinson’s disease and epilepsy. However, their effects in HD is largely unexplored. Neurodegenerative diseases share several common pathways and thus it is likely that a combination of selected fatty acids show neuroprotective effects in HD. This study utilized a cell line model of HD expressing inducible mutant huntingtin fragment with 150 polyQ repeats (HD150Q) to investigate neuroprotective effects of two medium chain fatty acids and one triglyceride. Significant reduction in mutant HTT aggregates and mitochondrial oxidative stress and restoration of ATP levels was observed upon treatment with Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate. Encouraging results in the cell line model opens avenues for investigating the underlying molecular mechanisms and validation in the animal models.

Keywords: Decanoic acid, 2-butyloctanoic acid, Glyceryl triacetate, Huntington’s disease, Mitochondrial dysfunction, Mutant huntingtin aggregates, Oxidative stress

Huntington's disease (HD) is a rare autosomal dominant neurodegenerative disorder, occurring with an incidence of 1 in 10,000 individuals and has no cure. The etiology of HD involves an abnormal expansion of CAG repeats in exon 1 of the huntingtin (Htt) gene that translates into expanded polyglutamine (polyQ) tract in mutant huntingtin (mHTT) protein resulting in alteration of Htt structure and facilitating abnormal protein-protein interaction and mHTT aggregation within neurons. These gain of function attributes of mHTT protein initiates a degenerative cascade, primarily affecting medium spiny striatal and cortical neurons, leading to dysfunction and subsequent neuronal loss, giving rise to neuromuscular manifestations such as involuntary movements, psychiatric symptoms, and cognitive decline. Mutant huntingtin expression leads to dysregulated gene expression, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, oxidative stress, glutamate-mediated excitotoxicity, and cell death in a cell-autonomous manner. Furthermore, perturbations in ATP, a vital energy source for cellular processes, are observed in HD, contributing to compromised neuronal function. Consequently, targeting the toxic effects of mHTT in neuronal context has been the primary focus of global research efforts.

Currently, HD lacks a definitive cure, and available treatments primarily focus on alleviating motor and psychological symptoms. Fatty acids have emerged as potential therapeutic agents due to their multifaceted roles in neuronal health and function. Notably, omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) have demonstrated neuroprotective effects in various neurodegenerative disorders, including Alzheimer’s and Parkinson’s diseases. While the neuroprotective role of PUFAs and MUFAs has received extensive investigation, the involvement of saturated medium-chain fatty acids (MCFAs) in neurodegenerative processes has gained recent attention.

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Decanoic acid (C10:0) and 2-butyloctanoic acid are specific saturated medium-chain fatty acids (MCFAs) that hold promise as neuroprotective agents in neurodegenerative diseases. Decanoic acid exhibits antiepileptic properties by inhibiting neuronal excitability and enhancing GABAergic neurotransmission\(^{20}\). Additionally, it demonstrates neuroprotective effects in Alzheimer's disease models through the enhancement of mitochondrial function\(^{21}\). 2-butyloctanoic acid, a derivative of Decanoic acid, possesses anti-inflammatory properties, modulating microglial activation and reducing proinflammatory responses in models of Parkinson's and Alzheimer's diseases\(^{22}\). The precise mechanisms underlying their neuroprotective effects are still under investigation. Nonetheless, their ability to modulate neuronal excitability, improve mitochondrial function, and regulate neuroinflammatory responses underscores their potential therapeutic applications in neurodegenerative disorders. Furthermore, the specific effects of fatty acids and their derivatives on mHTT aggregates, oxidative stress, and ATP levels in HD are currently not well characterized.

This study aims to investigate the potential neuroprotective properties of fatty acids and their derivatives in HD by utilizing a cell line model of Huntington's disease. Given that fatty acids are obtained from diet and are generally safe to consume, the neuroprotective effect may lead to development of novel therapeutics for HD. Understanding the underlying molecular mechanisms implicated in the neuroprotective effects of fatty acids could pave the way for targeted interventions and contribute to the ultimate goal of alleviating the burden imposed by Huntington's disease.

### Materials and Methods

#### Cell culture

The Neuro 2a cell line, expressing enhanced green fluorescent protein (e-GFP)-tagged truncated N-terminal huntingtin (Tn-HTT) with 150 polyglutamine (150Q) repeats under the regulation of an ecdysone-inducible promoter, was a kind gift from Prof. Nihar Ranjan Jana (National Brain Research Centre, Gurgaon, Haryana, India) and will be referred to as HD150Q. HD150Q cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cytiva HyClone, USA), supplemented with 10% heat-inactivated fetal bovine serum (Cytiva HyClone, USA) and 1% (v/v) penicillin-streptomycin antibiotic mixture (Gibco, Invitrogen). The cells were maintained at 37°C in a humidified 5% CO\(_2\) incubator. The HD150Q cell line represents a stable dual transfectant, comprising two plasmids: pVgRXR (conferring Zeocin resistance) and pND-HD. The selection of transgenic cells was accomplished by employing a concentration of 0.4 mg/mL Zeocin (InvivoGen) along with G-418 (Sigma-Aldrich). Notably, the HD150Q cells demonstrate expression of GFP-fused mutant huntingtin exclusively in the presence of Ponasterone A (Sigma-Aldrich).

#### Kits and reagents

Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate were procured from Sigma Aldrich. Anti-HTT antibody (Anti-HTT 4-19, N-Terminal) was procured from Coriell Institute for Medical Research (USA). Anti-Rabbit-HRP secondary antibody was obtained from Cell Signaling, USA. The ATP Determination Kit was acquired from Invitrogen, Thermo Fisher Scientific, USA, for the measurement of ATP levels. MitoSOX Red, a fluorescent dye for detecting mitochondrial superoxide, was purchased from Invitrogen, Thermo Fisher Scientific, USA.

#### Fluorescence microscopy

To visualize the aggregates of mutant huntingtin (mHTT) tagged with green fluorescent protein (GFP), HD150QQ cells were plated in 6-well plates (NEST) at a density of 0.3 × 10\(^6\) cells per well. The mHTT overexpression was induced by the addition of Ponasterone A (1 µM) for indicated time points. To investigate the impact of Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate on mutant huntingtin aggregation, cells were treated with indicated concentrations of fatty acids or DMSO (vehicle control) for 48 h. Subsequently, the cells were stained with Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, USA) and observed under an Eclipse Ti2-E inverted fluorescence microscope (Nikon, Japan). During the experiments, the gain, offset levels, and laser power of the detectors were meticulously calibrated and maintained at consistent settings. The quantification of mHTT puncta per field was performed manually and represented as average number of puncta per field.

#### Western blotting

HD150Q cells were seeded in 6-well plates at a density of 0.3 × 10\(^6\) cells per well and subjected to the specified treatments for a designated duration. Following treatment, the cells were collected, rinsed with ice-cold 1X phosphate-buffered saline (PBS), and then suspended in lysis buffer (50 mM Tris-Cl, pH 8.0,
and 0.1% Triton X-100) supplemented with 1X protease inhibitor cocktail (Sigma-Aldrich, USA) and lysed by sonication using a bath sonicator, employing 10s ON/OFF cycles for a total of 3 rounds. The protein in resulting cell lysates was quantified using the Bradford assay (Bio-Rad, USA), and equal amounts of proteins were loaded and subjected to western blotting as described previously. Briefly, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA) and blocked using a 5% blocking buffer (composed of 5% non-fat dried milk and 0.1% Tween-20 in TBS). The membrane was then incubated overnight at 4°C with anti-HTT antibody. After washing the membrane three times for 10 minutes each with 1X TBS containing 0.1% Tween-20 (TBST), the membrane was probed with an HRP-conjugated anti-rabbit secondary antibody (obtained from Cell Signaling, USA). Following another three washes with 1X TBST, chemiluminescent substrate was applied to the membrane, and the resulting blot bands were visualized using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, USA). The acquired blots were subsequently analyzed using Image Lab software (Bio-Rad Laboratories, USA). To determine the relative intensity of each protein's expression, ImageJ image processing software (developed by Rasband WS; NIH) was employed.

ATP levels
The impact of the administered fatty acid treatments on cellular ATP production was examined using a luminescent-based ATP determination kit (Molecular Probes/Life Technologies, Canada), following the manufacturer's guidelines. HD15Q cells were treated with 1 μM Ponasterone A and varying concentrations of the fatty acids for a duration of 24 h. Subsequently, the cells were washed with ice-cold 1X PBS and prepared for the assessment of the fatty acid treatments' influence on cellular ATP levels. The cells were lysed on ice using 0.1% Triton X-100, and a 10 μL aliquot of the resulting cell lysate was diluted in 100 μL of an ATP determination master mix comprising 25 mM Tricine buffer (pH 7.8), 5 mM MgSO4, 0.5 mM D-luciferin, 1.25 μg/mL firefly luciferase, 100 μM EDTA, and 1 mM DTT. The luminescence intensity was measured utilizing a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments). The protein content was quantified using the Bradford assay, and the ATP concentrations were normalized with their respective protein concentrations.

Mitochondrial ROS determination
Mitochondrial Reactive Oxygen Species (ROS) levels were assessed through the utilization of MitoSOX Red staining at a concentration of 5 μM. To initiate the experiment, HD15Q cells were seeded in 6-well plates at a density of 0.3 × 10^6 cells per well. The cells were then subjected to treatment with Ponasterone A and/or varying concentrations of the fatty acids, followed by staining with MitoSOX Red and Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, Canada). The nuclei were visualized using a fluorescence microscope, and the results were analyzed using ImageJ software.
USA). Subsequently, the cells were observed and analyzed under an Eclipse Ti2-E inverted fluorescence microscope (Nikon, Japan). The mean fluorescence intensity was quantified using ImageJ software, and the obtained data were further processed and plotted using GraphPad Prism 6 software.

**Statistical analysis**

GraphPad Prism: version 6 software was primarily employed for conducting various statistical analyses and generating graphical representations of the obtained results. The experimental data, concerning the number of observations, is expressed as mean ± SEM. In cases where a comparison was made between two groups with repeated measurements, the Student’s unpaired t-test was utilized to determine the statistical significance of each group. For experiments involving more than two groups, the one-way analysis of variance (ANOVA) test was employed, followed by Dunnett’s multiple comparison test to assess the level of significance. Each experiment was independently repeated a minimum of three times, and statistical significance was considered at a probability value of $P \leq 0.05$.

**Results**

**Decanoic Acid, 2-Butyloctanoic Acid, and Glyceryl Triacetate prevent mHTT Aggregation**

In this study, Fluorescence microscopy was employed to visually examine and quantify the presence of GFP-tagged mutant huntingtin (mHTT) aggregates in the HD150Q cells. The primary objective was to assess the ability of Decanoic acid, 2-Butyloctanoic acid, and Glyceryl triacetate to mitigate the formation of mHTT aggregates. Following a 48 h incubation period, fluorescence microscopy images displayed a notable decrease in the number and intensity of GFP-tagged mHTT aggregates in the Ponasterone A induced HD150Q cells treated with the fatty acids, in comparison to cells induced with Ponasterone A alone (Fig. 1A). This reduction was visually apparent, indicating a potential modulating influence of the tested fatty acids on mHTT aggregate formation. Subsequently, fluorescence microscopy images encompassing multiple fields were employed to quantify the average number of puncta per field. The results further substantiated the observed decrease in mHTT aggregates. Statistical analysis revealed a significant decline in the number of puncta following treatment with Decanoic acid, 2-Butyloctanoic acid, and Glyceryl triacetate (Fig. 1B).

Western blotting was carried out to validate the findings from fluorescence microscopy. To confirm the reduction of insoluble GFP-tagged mutant huntingtin (mHTT) aggregates following fatty acid treatment, HD150Q cells were incubated with either Ponasterone A (1 μM) alone or Ponasterone A plus fatty acids at indicated concentrations for 48 h. HTT Protein expression was assessed using an anti-huntingtin antibody. As illustrated in (Fig. 2), insoluble mHTT aggregates increased in cells treated solely with Ponasterone A (see upper panel), while soluble HTT gradually decreased (see lower
Interestingly, treatment with various concentrations of fatty acids (100 μM and 300 μM) prevented mHTT aggregation and kept the mHTT in soluble form, consistent with the fluorescence microscopy results.

Overall, the results obtained from fluorescence microscopy and western blotting analyses indicate a substantial reduction in GFP-tagged mHTT aggregates in HD150Q cells treated with Decanoic acid, 2-Butyloctanoic acid, and Glyceryl triacetate. These findings suggest the potential of these fatty acids as modulators for preventing and/or reducing mHTT aggregates in Huntington’s disease.

**Decanoic Acid, 2-Butyloctanoic Acid, and Glyceryl Triacetate abrogate Mitochondrial Reactive Oxygen Species (ROS) levels**

To evaluate the effect of Decanoic acid, 2-Butyloctanoic acid, and Glyceryl triacetate on mitochondrial ROS production, HD150Q cells were stained with MitoSOX Red and Mitochondrial ROS levels were visualized and quantified using fluorescence microscope. The stain specifically targets mitochondria and exhibits increased red fluorescence intensity upon oxidation by ROS, enabling reliable measurement of mitochondrial ROS levels. Microscopy images revealed a dramatic reduction in fluorescence signal in the Ponasterone A induced and fatty acid-treated HD150Q cells compared to the cell induced with Ponasterone A alone (Fig. 3A). Quantitative analysis confirmed the significant decrease in mitochondrial ROS levels, indicating the potential of Decanoic acid, 2-Butyloctanoic acid, and Glyceryl triacetate to decrease mitochondrial ROS production (Fig. 3B).
Discussion

Huntington's disease is a neurodegenerative disorder that typically manifests in late adulthood and is marked by a range of symptoms, including motor impairments, cognitive deficits, and psychiatric disturbances. Additional clinical manifestations include unintentional weight loss, disruptions in sleep patterns and circadian rhythms, as well as dysfunction of the autonomic nervous system. The absence of a definitive cure for Huntington's disease is widely acknowledged; however, numerous therapeutic interventions exist to alleviate symptoms and enhance the quality of life for affected individuals. Over the past two decades, a total of 99 clinical trials involving 41 distinct compounds have been conducted on patients with Huntington's disease. Regrettably, the progression rate from one stage to the next remains low, with only 3.5% of trials advancing to subsequent phases.

However, various molecules such as antisense oligonucleotides (ASOs) and small interfering RNA (siRNA) have reached different stages of human clinical trials, as reported by Travessa et al. (2017). Symptomatic treatment of HD involves the use of different medications, including tetrabenazine (TBZ) and deuterated TBZ (dTbZ), as highlighted by Dash and Mestre (2020). Nonetheless, the utilization of novel synthetic drugs is accompanied by significant side effects and high toxicity. An intriguing alternative approach involves the use of phytochemicals and/or dietary supplements instead of target-specific synthetic drugs for the treatment of neurodegenerative diseases, offering numerous advantages such as minimal side effects and low toxicity to normal cells. The exploration of natural compounds that target multiple cellular processes holds promise for the development of innovative treatment strategies for polyglutamine (polyQ) diseases.

In recent years, fatty acids have emerged as promising neuroprotective compounds and have been explored as therapeutic agents for the treatment of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Specifically, medium-chain fatty acids, including Decanoic acid, as studied by Damiano et al. (2020), and branched isomers of octanoic acid, such as 2-butyloctanoic acid and 2-butylopropanoic acid, have demonstrated potential in reducing drug-resistant epilepsy. These fatty acids have also been found to exhibit HDAC...
inhibition activity without teratogenic effects, as observed by Eikel et al. (2006)\textsuperscript{30}. Moreover, essential fatty acids and sulphur-containing fatty acids, such as Lipoic acid, have demonstrated neuroprotective effects against motor deficits and have been shown to significantly increase survival rates in transgenic mouse models of Huntington's disease\textsuperscript{31-33}. However, the precise mechanism by which these fatty acids exert their neuroprotective effects remains unclear. To investigate the neuroprotective effects of two specific medium-chain fatty acids, namely Decanoic acid, 2-butyloctanoic acid and Glyceryl triacetate, we employed an inducible cell line model expressing exon 1 of HTT with 150Q repeats.

The findings of this study provide compelling evidence supporting the neuroprotective properties of indicated molecules in an HD150Q cell line model representative of Huntington's disease. The characteristic build-up of mHTT aggregates, oxidative stress, and perturbed adenosine triphosphate (ATP) levels are critical pathological hallmarks associated with HD. Consequently, it is imperative to focus on these aberrant processes in order to devise effective therapeutic strategies for intervention in HD.

The experimental findings unequivocally established that the administration of Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate resulted in a significant decline in the formation of mHTT aggregates within HD150Q cells. These results align with previous investigation that has also reported the capacity to influence protein aggregation dynamics in the context of neurodegenerative disorders. Dirleise Colleet et al. (2012) demonstrated that interventions targeting mitochondrial dysfunction, a prominent characteristic of Huntington's disease, such as MK-801, exhibited a correlation with the observed reduction in mHTT aggregates observed in this current investigation\textsuperscript{34}. The observed decrease in mHTT aggregates induced by these fatty acids suggests their potential to impede the aggregation process itself or enhance the clearance of mHTT species from affected cells.

Moreover, the investigation revealed that Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate effectively mitigated oxidative stress by diminishing the production of reactive oxygen species (ROS) within the mitochondria. Oxidative stress, a pivotal factor in the pathogenesis of HD, substantially contributes to neuronal impairment and dysfunction\textsuperscript{35}. Previous studies have reported the antioxidant attributes of specific fatty acids and their derivatives, corroborating the present findings\textsuperscript{36,37}. For instance, Liberato et al. (2012) highlighted the selective peroxisome proliferator-activated receptor (PPAR) γ activation and antioxidant properties exhibited by medium-chain triglycerides, including 2-butyloctanoic acid\textsuperscript{36}. Similarly, Mett et al. (2021) demonstrated the neuroprotective effects of Decanoic acid in primary cortical neurons, reinforcing the observed amelioration of oxidative stress observed in the current study\textsuperscript{37}. The ability of Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate to mitigate mitochondrial ROS production underpins their potential as antioxidants or modulators of mitochondrial function. These findings align with earlier investigations that have elucidated the antioxidant characteristics of specific fatty acids and their derivatives\textsuperscript{36,37}.

Another notable finding of this investigation is restoration of ATP levels in HD150Q cells by the administration of fatty acids. ATP depletion is a hallmark of HD\textsuperscript{38} and contributes to the observed energy deficiency in affected neurons\textsuperscript{39}. The restoration of ATP levels observed with Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate suggests their potential to enhance mitochondrial function and stimulate ATP synthesis. This observation aligns with the study conducted by Chang et al. (2013), wherein medium-chain fatty acids associated with a ketogenic diet were shown to enhance seizure control through modulation of ATP production and energy metabolism\textsuperscript{39}. Furthermore, Chang et al. (2016) demonstrated that Decanoic acid can safeguard cortical neurons by improving energy metabolism and augmenting ATP production\textsuperscript{20}.

In summary, we provide a compelling evidence for neuroprotective potential of Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate in the HD150Q cell line model of Huntington's disease. The observed ability of these fatty acids to alleviate mHTT aggregates, oxidative stress, and ATP depletion establishes a foundation for subsequent exploration and the formulation of targeted therapeutic approaches for HD. However, it is important to acknowledge that this study relied on a cell line model, necessitating further research to corroborate these results in in vivo models and eventually in human clinical trials.

Comprehending the fundamental molecular mechanisms through which these fatty acids exert their neuroprotective effects is of paramount importance to facilitate their translation into clinical
applications. Subsequent investigations should prioritize the elucidation of the precise pathways and targets engaged by Decanoic acid, 2-butyloctanoic acid, and Glyceryl triacetate in the context of Huntington's disease.

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Conflict of interest
All authors declare no conflict of interest.

References


