FENUGREEK SEED EXTRACT (FSE) AND 4-HYDROXYISOLEUCINE (4OH) INDUCE SIRT3-MEDIATED ANTIOXIDANT EFFECTS IN DIABETIC C57BL/6 MICE BRAIN TISSUE IN VIVO

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Abstract— Diabetes is a detrimental metabolic disorder, associated with oxidative stress and mitochondrial dysfunction in the brain. This study was designed to evaluate the antihyperglycemic and antioxidant (AO) potential of Fenugreek seed extract (FSE) and 4-hydroxyisolecuine (4OH) in Normoglyceamic and Streptozotocin (STZ)-induced diabetic C57BL/6 mice brain tissue. Following a 10-day STZ (50mg/kg BW) diabetic induction, and 15-day FSE (100mg/kg BW) and 4OH (100mg/kg BW) treatment, the OGTT test revealed decreased blood glucose levels in diabetic mice, exhibiting their hypoglycaemic ability. Both FSE and 4OH displayed AO potential by increasing PGC-1α, phospho- and total Nrf2, Sirt3, and Pink1 protein levels. Furthermore, transcription of AMPKα1, Sirt3, NQO1, SOD1, and SOD2 genes were induced in treated mice brain tissue. In conclusion, we propose a mechanism by which FSE and 4OH exert hypoglycaemic effects in the brain through AO related mitochondrial responses.

Index Terms— Sirtuin 3, diabetes, antioxidant, brain

I. INTRODUCTION

Diabetes is a major noncommunicable disease with growing challenges worldwide, and a spectrum of related metabolic diseases. Increasing interest on diabetes and the brain has surfaced due to its association with neurological co-morbidities (Roriz-Filho et al., 2009). Insulin resistance accelerates complications such as glucose autooxidation, and reactive oxygen species (ROS). The antioxidant (AO) defences are impaired further exacerbating mitochondrial dysfunction (Rolo and Palmeira, 2006) in hyperglycaemic (HG) states.

Cellular energy metabolism is controlled through integration of regulatory circuits by Peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) (Kong et al., 2010). A positive feedback-loop exists between Sirtuin 3 (Sirt3), a mitochondrial deacetylase, and PGC-1α. An important target of Sirt3 is AMP-activated protein kinase (AMPK), through which mitochondrial oxidative phosphorylation (OxPHOS), glucose metabolism, and ATP levels are regulated (Ahn et al., 2008). Downstream regulation of transcriptional regulator nuclear respiratory factor 2 (Nrf2) is exerted by PGC-1α. Additionally, Nrf2 regulates the AO response element (ARE), promoting detoxification enzyme expression such as cytoplasmic superoxide dismutase 1 (SOD1), mitochondrial SOD2, and NQO1 (NADPH: quinone oxidoreductase 1) (Nguyen et al., 2009). Collectively, these transcription factors are proposed as therapeutic candidates for diabetic complications and neurodegenerative disorders (Xiong et al., 2015). Fenugreek (Trigonella-foenum-graecum L) belongs to the Papilionaceae section of the Leguminosae family (Basu and Srichamroen, 2010). 4-Hydroxyisoleucine (4-OHIle) is a branched-chain amino acid present in fenugreek seeds with antidiabetic effects through regulation of insulin resistance (IR) (Broca et al., 2004, Sauvaire et al., 1998). Fenugreek seed extract (FSE) is rich in phenols and saponins which contribute to its antioxidant effects in the liver and brain (Genet et al., 2002, Yoo et al., 2008). Here we aim to delineate the molecular mechanism of FSE and 4OH antioxidant potential in brain tissue of non-diabetic and STZ-diabetic mice. The present study may encourage the use of such effective alternative drugs in the treatment of neurological associated diabetic complications.

II. RESULTS

2.1. FSE and 4OH ameliorates insulin resistance in STZ treated C57BL/6 mice

Glucose tolerance was assessed in normoglycemic and STZ-induced HG mice using the oral glucose tolerance test (OGTT). The area under curve (AUC) was calculated as a measure of glucose excursion (Sakaguchi et al., 2016). Both FSE and 4OH showed improved glucose tolerance in comparison to controls

Table 1 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for Normoglyceamic group

Area under curve (AUC)	Control	FSE	4OH
AUC 1 (30-0 min)	217.5 ± 50*	199.5 ± 21#	183 ± 24+
AUC 2 (60-30 min)	222 ± 34*	202.5 ± 72#	187.5 ± 48+
AUC 3 (90-30 min)	223.5 ± 61*	201 ± 45#	185.25 ± 23+
AUC 4 (120-90 min)	221.25 ± 19*	207 ± 35#	184.5 ± 25+
Total AUC	**884.25 ± 124**	**810 ± 264**	**740.25 ± 249**

Data are shown as mean ± SD of 5 animals; *, #, + Values with different superscript letters within a row are significantly different from each group of animals (Tukey's multiple range posthoc test, $p < 0.05$). FSE: fenugreek seed extract, 4OH: 4-hydroxyisoleucine

Table 2 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for HG group

Area under curve (AUC)	Control	FSE	4OH
AUC 1 (30-0 min)	498 ± 29*	484.5 ± 17#	423 ± 17+
AUC 2 (60-30 min)	508.5 ± 54*	465 ± 89#	453 ± 18+
AUC 3 (90-30 min)	519 ± 45*	448.5 ± 16#	445.5 ± 19+
AUC 4 (120-90 min)	519 ± 34*	448.5 ± 10#	445.5 ± 15+
Total AUC	2044.5 ± 163*	1846.5 ± 257#	1767 ± 138

Data are shown as mean ± SD of 5 animals; *,#,+ Values with different superscript letters within a row are significantly different from each group of animals (Tukey's multiple range posthoc test, $p < 0.05$). FSE: fenugreek seed extract, 4OH: 4-hydroxyisoleucine

2.2. FSE and 4OH upregulate the deacetylase Sirt3 protein and gene expression

Sirt3 plays a crucial role in mitochondrial function. Diabetes associated OS is linked to alterations in Sirt3 expression (Jing et al., 2010). Here, we show that FSE and 4OH efficiently increases Sirt3 activity (Fig 1A) and mRNA expression (Fig 1B) in HG murine brain tissue.

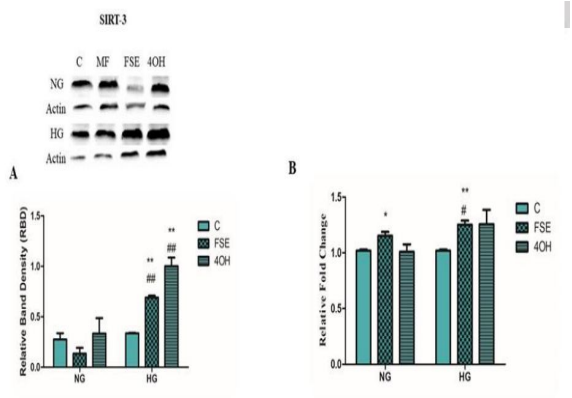


Figure 1: Sirt3 protein (A: **/ ## p<0.005) and mRNA (B: */# p< 0.05, ** p< 0.005) expression was increased in the HG group relative to the control in mice brain tissue (*: NG- FSE/4OH vs NG-C, NG- FSE/4OH vs HG-FSE/4OH)

2.3. 2.3 Mitochondrial regulator, PGC-1α and metabolic sensor gene expression induced by FSE and 4OH in diabetic mouse brain

Energy depletion and changes in catabolic rates such as those experienced in HG can be sensed by AMPK and Sirtuins (Rodgers et al., 2005). PGC-1α can restore metabolic homeostasis, we show the upregulation of its activity (Fig 2A), as well as AMPKα1 subunit (Fig2B) in brain tissue following FSE and 4OH treatment post STZ-diabetic induction.

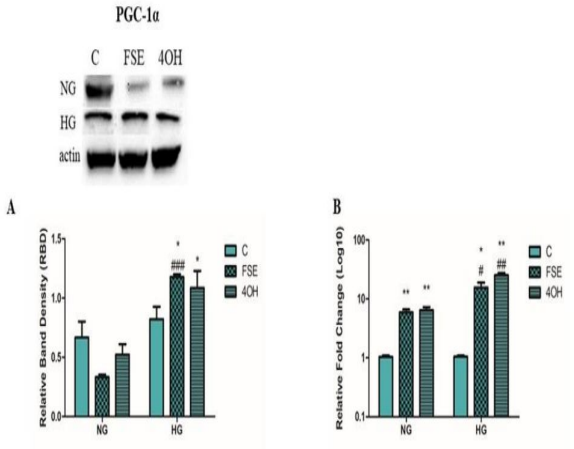


Figure 2: PGC-1α (A: * p< 0.05, ### p< 0.0001) and AMPKα1 (B: */# p< 0.05, **/## p< 0.005) assessment depicted increased expression following treatment in mouse groups (*: NG-FSE/4OH vs NG-C, NG- FSE/4OH vs HG-FSE/4OH)

2.4. 2.4 Activity and expression of Nrf2 is upregulated following FSE and 4OH

Aberrations in Nrf2 signalling causes neuronal susceptibility to OS (Lee et al., 2003). As indicated by protein activity (Fig 3), Nrf2 levels were increased in diabetic mouse brain exposed to FSE and 4OH in vivo.

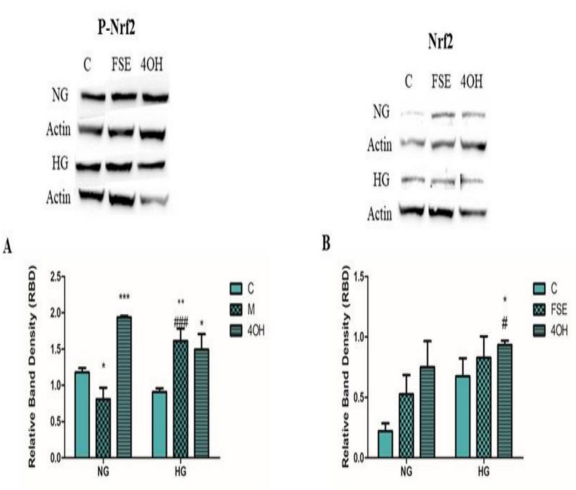


Figure 3: Treated mice brain tissue exhibited higher activity of Nrf2 (A: *p<0.05, **p<0.005, */### p<0.0001). Further increased Nrf2 expression in mice brain tissue was noted after FSE and 4OH post HG induction (B: */# p< 0.05) (*: NG-FSE/4OH vs NG-C, NG- FSE/4OH vs HG-FSE/4OH)**

2.5. AO defence enzymes induced by FSE and 4OH

The ARE is transcriptionally involved in the activation of genes coding for several antioxidant proteins and phase 2 detoxification enzymes. Results show that FSE and 4OH treatment induced slight upregulation of NQO1 (A), SOD1 (B), and SOD2 (C) (Fig 4). Thus, suggesting their ability to maintain AO defences in the brain a diabetic state.

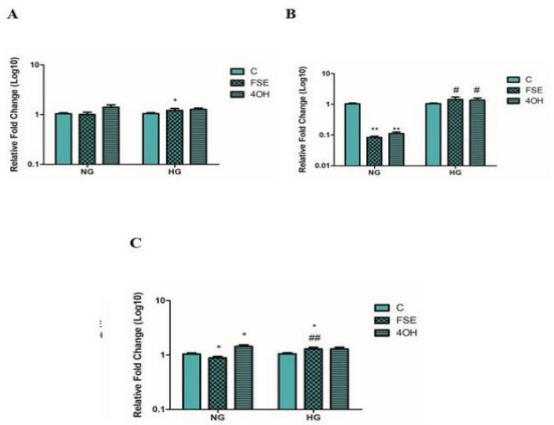


Figure 4: FSE promotes NQO1 (A: *p<0.05), SOD1 (B: **p<0.005, #p<0.05), and SOD2(C: *p<0.05, ##p<0.005) enzyme activity more efficiently than 4OH in brain tissue of diabetic mice. (*: NG- FSE/4OH vs NG-C, NG- FSE/4OH vs HG-FSE/4OH)

2.6 FSE and 4OH regulate mitochondrial dysfunction by mediating PINK1 in diabetic mouse brain tissue

OS induced mitochondrial damage is allied with Pink1 recruitment of Parkin enabling clearance of damaged mitochondria (Xiao et al., 2017). FSE did not induce Pink1 activity, whereas 4OH efficiently upregulated its expression (Fig 5) as compared to control following STZ-diabetic induction in mice.

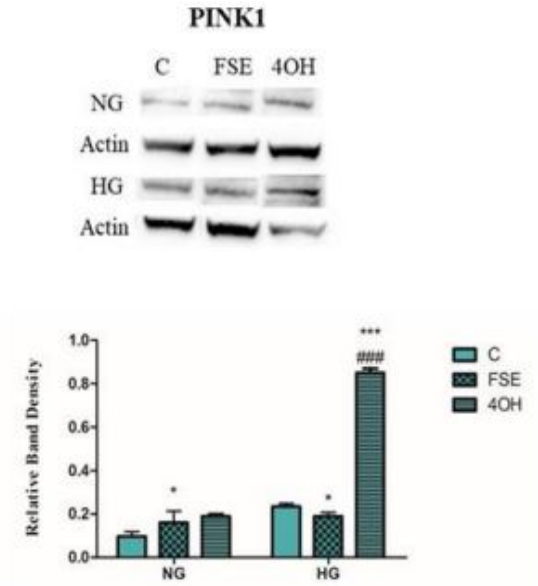


Figure 5: 4OH induced Pink1 transcription following 15day treatment (* p< 0.05, */### p< 0.0001) (*: NG- FSE/4OH vs NG-C, NG- FSE/4OH vs HG-FSE/4OH)**

III. DISCUSSION

Hyperglycaemia increases reactive oxygen species (ROS) production and mitochondrial dysfunction which are involved in metabolic disorders. Sirtuin 3 (SIRT3) is a primary mitochondrial deacetylase that regulates mitochondrial function and antioxidant (AO) defence (Jing et al., 2010). We investigated the role of SIRT3 in AO defence under STZ-induced hyperglycaemic conditions in the brain of C57BL/6 mice treated with FSE and 4OH.

Diabetes promotes the production of ROS leads to various pathological conditions including neurodegeneration, Alzheimer's and dementia (Butterfield et al., 2014). Neuronal cells have developed a network of regulated stress-response mechanisms. These include genes encoding components involved in managing OS in this network. Sirtuins could be considered among those genes as they are sensors of redox state (Webster et al., 2012). Sirt3 resides primarily in the mitochondria and has been shown to bind and deacetylate several metabolic and respiratory enzymes that regulate important mitochondrial functions (Ahn et al., 2008). Our study showed increased expressions of Sirt3 (Fig 1) in the HG group post FSE and 4OH treatment. Interestingly, the expression was much higher in NG mice treated with FSE and 4OH.

Sirt3 expression is required for the induction of AO genes by PGC-1α which may play a major role in the metabolic adaptations to energy metabolism in different tissues (Cantó and Auwerx, 2009). PGC-1α is a transcriptional co-activator that is activated in response to OS and plays an important role in regulating mitochondrial function and AO defence enzymes such as SODS (detoxifies superoxide radicals that are formed as a by-product of OXPHOS) (Lu et al., 2010). In an oxidative environment, PGC-1α induces Sirt3 expression through activation of ERR- α and in turn, Sirt3 stimulates PGC-1α expression through p-CREB thereby forming a positive feedback cycle and increasing AO defence (Brenmoehl and Hoeflich, 2013). Our data shows that both PGC-1α (Fig 2A), SOD1 and SOD2 (Fig 4A and B) expression is positively regulated by FSE and 4OH under HG conditions. Additional to regulating AO defence by PGC-1α, FSE and 4OH enables Sirt3 to detoxify ROS through deacetylation and activation of SOD2. Over-expression of Sirt3 was shown to reduce ROS in an Alzheimer's disease mediated stress hippocampal cell line (Weir et al., 2012). During HG states when energy supply is not limited, AMPK activity is prevented by the high intracellular ATP levels. We show that FSE and 4OH effectively promoted mRNA expression of the catalytic subunit, AMPKα1 (Fig 2B). It is known that AMPK directly phosphorylates PGC-1α, thereby inducing its activation (Jäger et al., 2007). Our results show that the improved AO response of FSE and 4OH in mice brain tissue involves AMPK-mediated activation of Sirt3 through a PGC-1α mechanism in vivo.

A more vigorous antioxidant response is induced by the Nrf2 transcription factor (Nguyen et al., 2009). Under HG-induced oxidative stress Nrf2 is released from its repressor, KEAP-1, and allowed to translocate to the nucleus (Yang et al., 2009). Here, it transcribes for several genes involved in AO response and ultimately neuroprotection (Sandberg et al., 2014). NQO1 forms part of the ARE and its

expression is induced by electrophilic metabolites and oxidative stress (Wu et al., 2013). FSE significantly increased gene expression of NQO1 in HG brain tissue as compared to the control (Fig 4A).

Nrf2 also plays a pivotal role in mitochondrial maintenance by stimulating the removal of damaged and defective mitochondria through up-regulation of Pink1 (Holmström et al., 2016). Antioxidant effects through Pink1 pathway have been shown in a knockout Pink1 mouse model of Parkinson's disease (Dinkova-Kostova et al., 2015). Interestingly, 4OH significantly increased Pink1 expression, indicating its potent antioxidant ability following STZ-diabetic induction (Fig 5). Previous studies indicate the AO effects of FSE are associated partly to the presence of flavonoids and polyphenols (El-Soud et al., 2007). Our study agrees with this and provides evidence of Nrf2-mediated AO roles of FSE and 4OH in NG and STZ-induced diabetic mice brain tissue.

CONCLUSION

The present findings indicate that the FSE and 4OH exert their anti-diabetic and neuroprotective effects, potentially through decreased HG and OS thereby ameliorating the adverse metabolic effects on the mitochondria and aid management of diabetic neuronal complications.

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