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4-Hydroxyisoleucine improves insulin resistance by promoting mitochondrial biogenesis and act through AMPK and Akt dependent pathway

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Abstract

4-Hydroxyisoleucine (4-HIL) is an unusual amino acid isolated from Fenugreek seeds (*Trigonella foenum* graecum L). Various studies have shown that it acts as an antidiabetic agent yet its mechanism of action is not clear. We therefore investigated the effect 4-HIL on the high fructose diet fed streptozotocin induced diabetic rats and L6 myotubes. 4-HIL (50mg/kg) has improved blood lipid profile, glucose tolerance and insulin sensitivity in a diabetic rat model. It has increased the glucose uptake in L6 myotubes in AMPK-dependent manner and upregulated the expression of genes (PGC-1α, PGC-1β, CPT 1 and CPT 2), which have role in mitochondrial biogenesis and energy metabolism in liver, skeletal muscles as well as in L6 myotubes. Interestingly, it also increased the AMPK and Akt expression along with their phosphorylated forms in liver and muscle tissues of treated animals. Altogether we concluded that 4-HIL act to improve insulin resistance by promoting mitochondrial biogenesis in high fructose diet fed STZ induced diabetic rats.

Key Words: 4-Hydroxyisoleucine, glucose tolerance, Insulin resistance, L6 myotubes mitochondrial biogenesis.

1. Introduction:

The pathogenesis of Type 2 diabetes mellitus is not well understood but there are growing evidences that metabolic abnormalities either due to dietary changes or lifestyle modulations leads to develop in to insulin resistance and Type 2 diabetes mellitus (Alberti and Zimmet, 1998,Tuomilehto, Lindström, Eriksson et al., 2001). In past few decades, consumption of high-energy diets such as diets rich in carbohydrates and fat has increased. These habits leads in to development of hypertriglyceridemia or increased plasma free fatty acid and ultimately results in to decrease in insulin mediated

glucose disposal by the muscles or insulin resistance (Kelley, Mokan, Simoneau et al., 1993). Diets rich in fructose have been shown to develop hypertriglyceridemia, obesity and insulin resistance in both human and rodents (Bezerra, Ueno, Silva et al., 2001,Zavaroni, Sander, Scott et al., 1980). According to some other studies, only fructose feeding for a long period develops nutritional tolerance in spite of developing insulin resistance and impaired glucose tolerance (Stark, Timar and Madar, 2000). These dietary modulations have greatly increased the risk for development of diabetes as well as other metabolic abnormalities. With this increasing risk of development of diabetes, search for new drug molecule for its management has become an urgent need.

Due to various undesirable side effects of available medications, use of traditional medicines provide an alternate source for the management of Insulin resistance and diabetes, second they are discovered from the natural products so they do not bear such side effects (Smyth and Heron, 2006,Koehn and Carter, 2005). There are various compounds and molecules have been identified from traditional medicinal plants and used as potential antidiabetic agents (Bailey and Day, 1989,Ocvirk, Kistler, Khan et al., 2013). *Trigonella foenum-graecum* is one of such plant known for its antidiabetic properties. It is commonly known as fenugreek, Seeds of the plant are being used as hypoglycaemic agent in treatment of diabetes from ancient time (Sharma and Raghuram, 1990). Extracts obtained from fenugreek are known to have potential antihyperglycemic and antidyslipidemic properties as studied in different animal models (El-Soud, Khalil, Hussein et al., 2007,Zia, Hasnain and Hasan, 2001,Al-Habori and Raman, 1998).

4-hydroxyisoleucine (4-HIL) is an unusual amino acid isolated from *Trigonella foenum-graecum,* act as antidiabetic and antidyslipidemic agent in diabetic subjects (Sauvaire, Petit, Broca et al., 1998). It is found to have insulinotropic properties, and in L6 myotubes 4-HIL increase 2-DG uptake and GLUT4 translocation to the cell surface without changing the total amount of GLUT4 and GLUT1 (Broca, Gross, Petit et al., 1999,Jaiswal, Maurya, Venkateswarlu et al., 2012). In the current study we have used high fructose diet fed low dose STZ induced diabetic rats and L6 myotubes for studying the effect of 4HIL as well as to explore its underlying mechanism of action.

Treatment with 4-HIL improves hyperglycaemia, insulin sensitivity and lipid profile of diabetic rats. Expression of both AMPK and pAMPK along with AKT and pAKT was found to be increased in liver and muscles of treated animals. We further identified that expression of key mitochondrial metabolic as well as regulator genes were upregulated in 4-HIL treated animals.

2. Materials

2.1. Chemicals and reagents

Fructose, casein, Cholesterol was purchased from Sisco Research Laboratory (Maharashtra, India.). The insulin Elisa kit was purchased from Mercodia (Uppsala, Sweden). Huminsulin 30/70 from Eli Lilly (India). c-DNA synthesis kit and Syber green were from Applied Biosystem (Foster City, CA). Primers were purchased from Integrated DNA Technologies (USA). Mouse anti-p-AMPK, mouse anti AMPK, anti GLUT-4, mouse anti p-AKT and anti AKT monoclonal antibodies were purchased from Cell Signaling Technology (USA). Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham Pharmacia Biotech (USA). All other chemicals were purchased from Sigma Aldrich (USA).

2.2. General Chemistry

Melting points were recorded on Buchi-530 capillary melting point apparatus and are uncorrected. IR spectra were recorded on Perkin-Elmer AC-1 spectrometer. $_1$H NMR spectra were recorded on Bruker Avance DPX 200 FT, Bruker Robotics and Bruker DRX 300, spectrometers at 300 MHz (^{1}H) and 75 MHz (^{13}C). Experiments were recorded in D_2O at 25 ºC. Chemical shifts were given in parts per million (ppm) downfield from internal standard Me_4Si (TMS). ESI mass spectra were recorded on JEOL SX 102/DA-6000. Chromatography was executed with silica gel (230–400 mesh) using mixtures of methanol and ethylacetate as eluants. Commercially available solvents were without further purification.

2.3. Isolation of 4-hydroxyisoleucine

The seeds (5 kg) of *T. foenum-graecum* were collected from the local market of Lucknow and extracted with 4 L of ethyl alcohol four times in a percolator. The resultant alcoholic extracts were (16 L) combined and concentrated under reduced pressure to give 100 g of alcohol extract. This was fractionated with chloroform and n-butanol successively. The resultant aqueous fraction (10 g) was subjected to conventional silica gel column chromatography using ethyl acetate and methanol (90:10) solvent system to give amino acid (750 mg). The compound purity was monitored on thin layer chromatography plates made of Silica gel 60 F_{254} in solvent system, BAW (Butanol: Acetic acid: water; 4: 1 : 5, v/v/v), which has shown Rf of 0.60. It was characterized as 4 hydroxyisoleucine by using ^{1}H NMR, ^{13}C NMR, IR and mass spectral data and comparing with literature data (Fowden, Pratt and Smith, 1973,Alcock, Crout, Gregorio et al., 1989). The HPLC analysis of the isolated compound indicated the presence of minor isomer, that is, 2R, 3R, 4S (6%) along with major isomer 2S, 3R, 4S (94%).

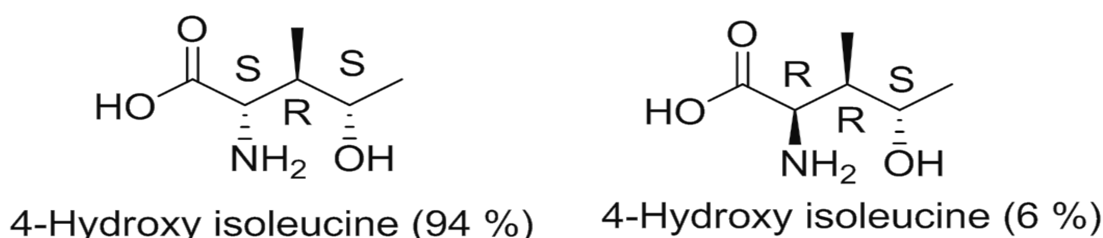

Fig.1. Chemical Structures of 4-Hydorxyisoleucine (isomeric mixture) isolated from *Trigonella foenum-graecum*

MP: 224-225 °C; IR: 3139, 1632 cm^{-1}, ^{1}H NMR in D_2O:δ 0.93 (3H, d), 1.22 (3H, d), 1.89 (1H, m), 3.85 (2H, m); ^{13}C NMR in D_2O: δ 14.9 (CH_3), 23.51 (CH_3), 44.1 (CH), 59.74 (CH), 72.64 (CH), 176.5 (CO); FAB Mass 148 (M+1).

2.4. Development of diabetic animal model

Male Sprague dawley rats of 3 to 4 weeks age group and body weight 120±12gm (n=40) gm were procured from the animal colony of Central Drug Research Institute,

Lucknow India. The study was approved by the Animal Ethics Committee formed by the Government of India in 1964. Animals were maintained in a temperature controlled room (22°C) on a 12-h light-dark wheel with free access to water and food. After acclimatization of 4 days they were divided into two dietary groups, one group was kept on a normal diet and other was kept on the high fructose diet (fructose 60% w/w) for the next 6 weeks. The composition of high fructose diet was taken as reported in previous studies with few modifications **(Table 1)** (Bezerra, Ueno, Silva et al., 2000)**.** Animals fed on high fructose diet were given an intra peritoneal injection of streptozotocin (30 mg/kg body weight) and continued feeding on fructose rich diet for next two weeks.

Table 1
Experimental diet Composition#

Ingredients	Normal Diet	High Fructose Diet
	g/Kg	
Fructose	-	600
Casein	210	210
Cholesterol	-	10
Fat	50	50
Carbohydrate	500	30
Corn starch	220	80
Mineral salt	10	10
Vitamins	10	10

 # The diet was prepared by the nutrition unit of national laboratory and animal centre, CSIR-Central Drug research Institute, India.

2.5. Study design:

2.5.1. Animal Experimentation design

After confirmation of insulin resistance and diabetes in Insulin tolerance test (ITT) and Oral glucose tolerance test (OGTT) experiments, diabetic animals were divided into three groups. One diabetic and one normal group was kept as control group received only 1% gum acacia whereas one diabetic group received fenofibrate (50 mg/kg) and another group received 4-HIL (50 mg/kg) for a period of 2 weeks. Dose of 4-HIL was taken as used in some previous studies. During treatment OGTT and ITT was done at weekly intervals and at the conclusion of the study blood samples were collected from anestesized animals from their retro orbital plexus. Tissue samples were collected by sacrificing the overnight fasted animals and stored accordingly for further studies.

2.5.2. Cell culture

L6 muscle cells were maintained in DMEM (D5648, sigma Aldrich) containing 25 mM glucose, 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (100 units/ml penicillin G, 10 g/ml streptomycin, and 25 mg/ml amphotericin B) in an atmosphere of 5% CO2 at 37°C. For differentiation myoblast fusion into myotubes cell culture medium was replaced with DMEM containing 2% (v/v) FBS at a density of 104 cells/ml for 6-8 days till the cells become ready for experiments (Sawada, Kawabata, Yamashita et al., 2012).

L6 myotubes were treated with 4-HIL at 10μM for 12 hours then 2-Deoxy glucose uptake was done in accordance with a prior studies (Jaiswal, Maurya, Venkateswarlu et al., 2012). Prior to treatment with 4-HIL cells were maintained in serum free DMEM for 2 hours then incubated with compound C (10μM) for 1 hour after that removed the medium and treated the cells with 4-HIL. For gene expression analysis total mRNA was isolated from the cells by TRIzol method rest of the study was done as described earlier. Similarly Immunoblotting analysis was done from the proteins isolated from the cells treated with 4-HIL and compound C by using Repa buffer in different set of experiments.

2.6. Oral Glucose tolerance test:

Oral glucose tolerance tests (OGTTs) were performed at weekly intervals as described earlier (Kwon, Kim and Kim, 2008). Animals were fasted for 12-h, thereafter fasting blood glucose was measured by sampling the blood from cut tail vein using Accuchek glucometer. D-glucose at a dose of 3g/kg was administered by oral gavage then blood glucose was measured at 30, 60, 90 and 120 min. post glucose administration.

2.7. Insulin Tolerance test:

Insulin tolerance test (ITT) was done in randomly fasted animals as reported earlier with little modifications (Weksler-Zangen, Raz, Lenzen et al., 2008). In brief blood glucose of randomly fasted animals were recorded from the tail vein by using glucometer and recorded as 0 min. reading then Insulin was given intraperitoneal at a dose of 1U/ kg body weight. Blood glucose was measured at 30, 60, 90 and 120 min post insulin administration and data was analyze to calculate the blood glucose AUC on PRISM 3 software.

2.8. 2-Deoxyglucose (2-DG) uptake assay

Glucose uptake was assessed by according to previously reported method in brief L6 myotube incubated for 5 min in HEPES-buffered saline [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO4, 1 mM $CaCl_2$ (pH 7.4)] containing 10 μM 2-DG (0.5 μCi/ml 2-[^{3}H] DG) at room temperature. Subsequently cells were rinsed with an ice-cold solution containing 0.9% NaCl and 20 mM D-glucose. To quantify the radioactivity incorporated, cells were lysed with 0.05 N NaOH and lysates were counted with scintillation fluid in a β-counter (Bekman LS-6500) (Sawada et al., 2012).

2.9. Biochemical analysis

On completion of the study, animals were fasted for 12-h before and blood samples were collected in EDTA coated vials by puncturing retro orbital plexus. Plasma was separated by centrifugation of blood at 3,000 rpm for 10 minutes then subjected to the analysis of lipid profile i.e., triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol on fully automated Cobas Integra 400 plus clinical analyser. Plasma insulin was measured by using insulin Elisa kit supplied by Mercodia, Sweden. Assay was performed according to the manufacturer protocol provided with kit.

2.10. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the frozen tissues using the TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA yield was quantified on Nanodrop 1000 and the RNA purity was determined based on the *A*260/*A*280 ratio. Reverse transcription of 2μg RNA was carried out according to the instructions of Prime Script TM 1st Strand cDNA Synthesis Kit. Reverse transcription was performed by using BioRad thermal cycler system and Quantitative PCR was performed by using SYBER Green QPCR Master Mix and Roche light cycler 480 system. The relative expression of the gene of interest was calculated by previously described method using the expression 2_DCT and reported in arbitrary units (Livak and Schmittgen, 2001).

2.11. Immunoblotting Study

Immunoblotting studies were done as described previously (Li, Xiong, Hu et al., 2009). In Brief protein samples were extracted from tissues in protein isolation buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, and 50 mM Tris·HCl) containing a protease and phosphatase inhibitor cocktail, and quantified by Bradford method. Equal amounts of protein was separated on a 4–12% polyacrylamide gel and subsequently transferred to polyvinylidene fluoride membranes (Millipore) using a semidry transfer cell (Bio-Rad, Hercules, CA). After transfer of protein on membranes they were blocked in Phosphate buffer saline Tween-20 (PBST) containing 5% non-fat dry milk. After one hour membranes were washed with PBST several times at swinging rocker then incubated overnight at 4^0C with primary antibodies for desired protein. After further washes with PBST, membranes were incubated with horseradish peroxidise conjugated secondary antibody for one hour at room temperature later on blots were washed with PBST and visualized by enhanced chemiluminescent substrate. Membranes were stripped and re-blotted with anti-Actinin-1and anti-β-Actin antibodies accordingly.

2.12. Statistical analysis

The results are expressed as mean±SEM or mean±SD. Data was analyzed on Prism 3 software using unpaired Student's t-test, used for analyzing the data between two groups where as one-way ANOVA followed by multiple comparison test (dunnett test) for more than two groups. A value of $p<0.05$ and less was considered statistically significant.

3. Results:

3.1. 4-HIL treatment improves the lipid profile but do not affect body weight of diabetic rats

High fructose diet feeding caused significant increase in body weight (281.5±20.4) than normal diet fed animals (190.3±11.5) **(Fig. 2A).** No significant difference was observed in body weight of treated group (254.2±12.7) and untreated

control group (262.2±15.8) **(Fig. 2B).** Lipid profile analysis showed marked improvement in triglyceride, cholesterol, LDL-C and HDL-C levels of the treated group of animals **(Fig. 2C)**. Plasma triglyceride, plasma cholesterol, and plasma LDL-cholesterol was decreased by 29.0% ($p<0.01$), 21.2% ($p<0.01$) and 29.2% ($p<0.05$) respectively whereas plasma HDL-C was found increased by 19.7% ($p<0.05$) in 4-HIL treated group of animals. Plasma insulin level was improved by 22.0% ($P<0.05$) in 4-HIL treated group in comparison to diabetic control **(Fig. 2D).** In fenofibrate treated group Plasma triglyceride, plasma cholesterol, and plasma LDL-cholesterol was decreased by 31.6% ($p<0.01$), 26.6% ($p<0.01$) 17.8% ($p<0.05$) respectively and plasma HDL-C was found increased by 6.7% ($p<0.01$) whereas plasma insulin was found decreased by 13.8% (not significant).

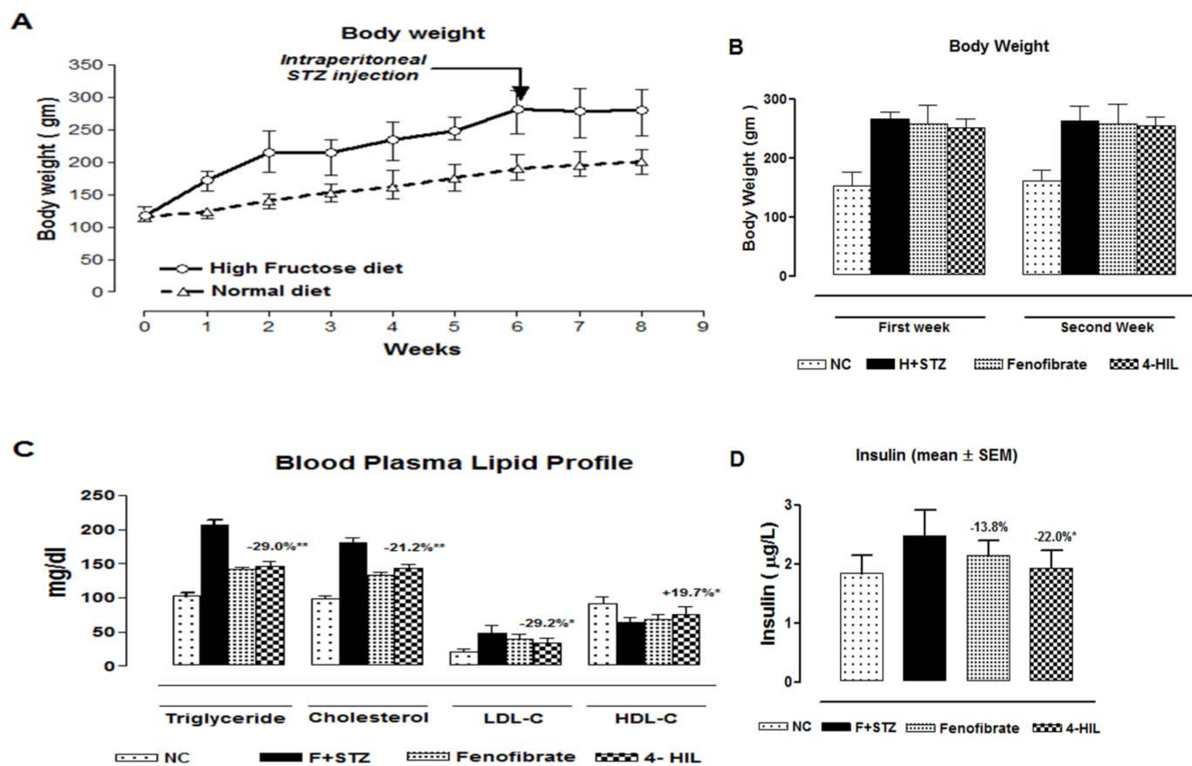

Fig.2. Effect of High Fructose Diet feeding and 4-HIL treatment on **A.** Body weight gain of animals during six weeks period of High Fructose Diet feeding and streptozotocin injection (n=50); **B.** Body weight of animal during two weeks of treatment (n=8); **C.** Plasma lipid profile of treated animals (n=8) and **D.** Plasma Insulin level (n=8). High Fructose Diet and streptozotocin induced diabetic control (F+STZ), 4-Hydroxyisoleucine (4-HIL), Normal control (NC). Graph show mean ± SE values of different groups. Significance between groups * $p< 0.05$, ** $p< 0.01$ on applying one way ANOVA followed by Dunnett test.

3.2. Effect of 4-HIL treatment on OGTT, ITT and fasting blood glucose

Six weeks of high fructose diet feeding and intraperitoneal injection of STZ causes impairment of glucose tolerance, which was shown by the untreated diabetic group in OGTT. During OGTT fasting blood glucose of diabetic group was found markedly higher than normal diet fed group as well as both treated groups i.e., fenofibrate and 4-HIL. When challenged with an oral glucose load diabetic control

animals as well as 4-HIL treated group of animals showed marked rise in blood glucose. In diabetic animals blood glucose was cleared less efficiently than 4-HIL treated groups. Blood glucose AUC was improved by 24.9% (p<0.01) and 29.9% (p<0.01) in 4-HIL treated group and in fenofibrate treated group it was found improved by 36.6% (p<0.01) and 34.3% (p<0.01) respectively during two weeks of OGTT **(Fig. 3 A & B).**

ITT was performed after two weeks of treatment. During ITT blood glucose of diabetic control was found 234.1±11.7 mg/dl and 216.6±10.5 mg/dl between two time intervals i.e., 15' and 60' post insulin administration without marked difference whereas in 4-HIL treated group of animals it was found 162.3±9.8 mg/dl and 126.0±7.2 mg/dl with marked difference at the same time interval **(Fig. 3C).** Fasting blood glucose of treated animals was also found improved by 23.7% (p<0.01) and 32.0% (p<0.01) in 4-HIL treated group whereas it was found improved by 33.2% (p<0.01) and 29.4% (p<0.01) in fenofibrate treated group respectively during two weeks of treatments **(Table 2).**

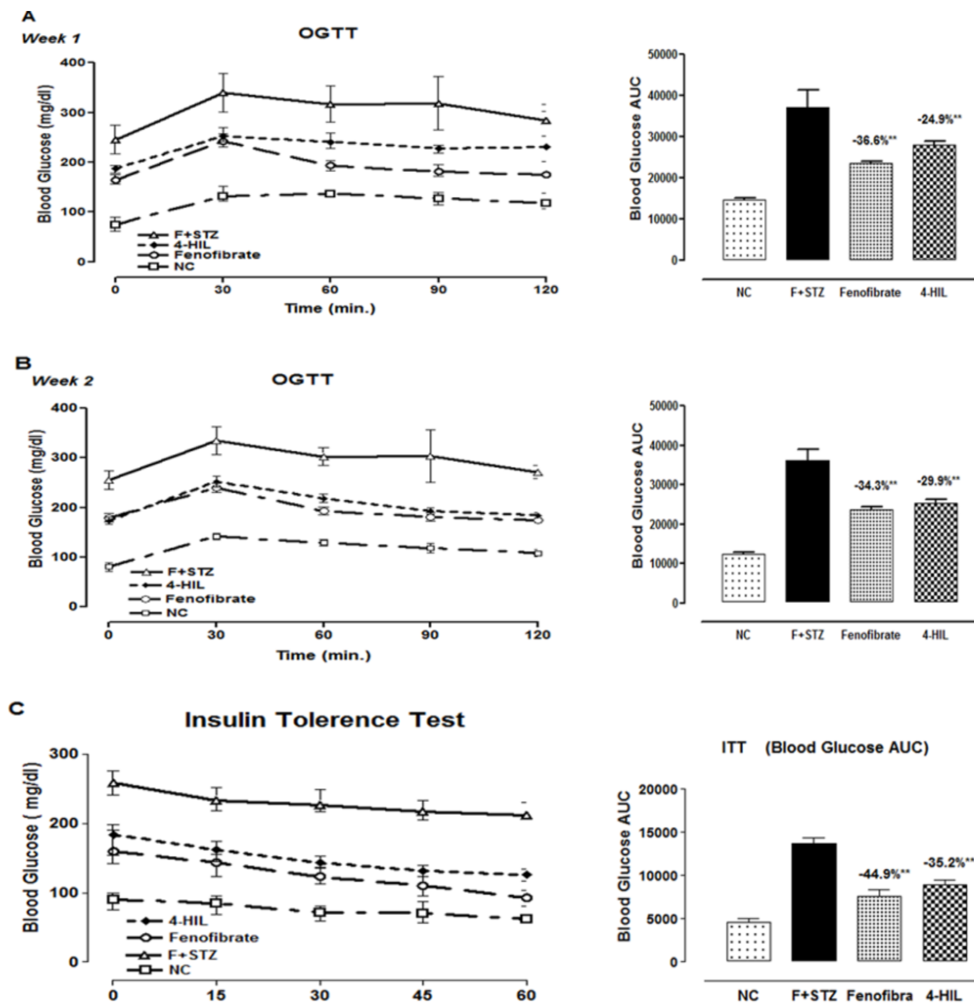

Fig.3. Effects of 4-HIL treatment on oral glucose tolerance test (A.& B) and Insulin tolerance test (C) of diabetic rats (n=8). High Fructose Diet and streptozotocin induced diabetic control (F+STZ), 4-Hydroxyisoleucine (4-HIL), Normal control (NC). Values are expressed as mean ± SD. Significance between groups * p< 0.05, ** p< 0.01 on applying one way ANOVA followed by Dunnett test.

Table 2

Effect of 4-HIL treatment on fasting blood glucose of High fructose diet fed STZ induced diabetic rats

S. No.	Animal Group	Fasting blood Glucose level mg/dl (Mean ± SD)	
		Week 1	Week 2
1	Normal Control	74.8 ± 12.5	80.8 ± 8.6
2	HFD Control	245.4 ± 29.3	255.4 ± 18.3
3	Fenofibrate	164.0±6.6**	180.0±8.3**
4	4-Hydroxyisoleucine	187.2 ± 6.1**	173.6± 7.4**

Values are expressed as mean ± SD (n=8). Significance between groups # NS (Not significant), * p< 0.05, ** p< 0.01,*** p< 0.001 on applying one way ANOVA followed by Dunnett test.

3.4. Glucose uptake by L6 myotubes treated with 4-HIL increases

4-HIL increases glucose uptake in L6-myotubes by 87.3% (p<0.01) and In presence of AMPK inhibitor compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyyrazolo [1,5-a] pyrimidineglucose uptake was found increased by 20.3% (p<0.5). Insulin alone increased the glucose uptake by 47.8% (p<0.01) but in presence of compound C glucose uptake was increased by only 5.4% (NS). Compound C alone inhibited the glucose uptake by 28.6% (p<0.05) **(Fig.4).**

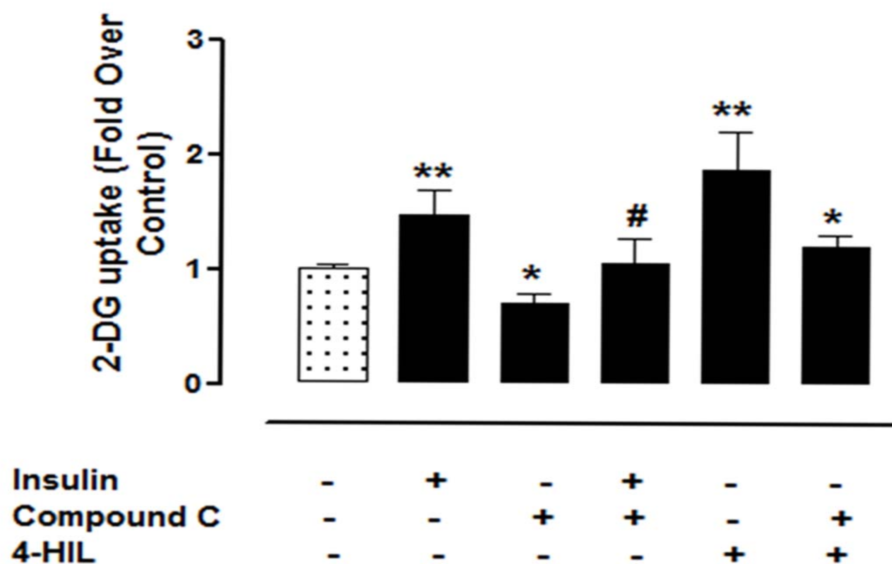

Fig.4. Improvement in 2-deoxy glucose uptake of L6 myotubes in different sets of experiments treated with 4-Hydroxyisoleucine (4-HIL), Compound C and Insulin. Significance between groups # NS (Not significant), * p< 0.05, ** p< 0.01,*** p< 0.001 on applying one way ANOVA followed by Dunnett test.

Table 3: Differentially expressed genes in liver and muscle of 4-HIL treated diabetic animals compared with untreated diabetic control group

S. No.	Gene Name	Gene Bank abbreviation	Accession no.	Fold Change in gene expression of Liver RNA		Fold Change in Gene expression of Muscle RNA	
				Mean	±SD	Mean	±SD
1	Transcription factor A	Tfam	NM_031326.1	2.0*	0.4	3.4*	0.4
2	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1a	NM_031347.1	5.2**	0.9	2.8*	0.5
3	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	Ppargc1b	NM_176075.2	8.1**	1.2	3.3	0.5
4	Peroxisome proliferator activated receptor gamma	Pparg	NM_013124.3	1.1	0.9	-1.5*	0.2
5	Peroxisome proliferator activated receptor alpha	PPAR α	NM_013196.1	0.8	0.5	-2.1	0.4
6	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	Atp5f1	NM_134365.2	7.1*	1.7	3.6*	0.8
7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	Ndufa10	NM_199495.2	2.2	0.5	1.3	0.8
8	Succinate dehydrogenase complex, subunit A, flavoprotein	Sdha	NM_130428.1	3.3*	0.5	2.4	0.7
9	Cytochrome c oxidase	Cox6b1	NG_028330.1	4.8*	1.2	2.3**	0.7
10	Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	NM_012751.1	3.1	0.11	2.1	0.12
11	Solute carrier family 2 (facilitated glucose transporter), member 3	Slc2a3	NM_017102.2	2.9*	0.07	3.1**	0.33
12	Glucose transporter-3	GLUT3	U17978.1	2.0*	0.12	1.9	0.21
13	Nuclear respiratory factor 1	Nrf1	NM_001100708.1	9.6**	1.8	2.5**	0.6
14	Acetyl-CoA carboxylase alpha	Acaca	NM_022193.1	ND		2.4	0.9
15	Acetyl-CoA carboxylase beta	Acacb	NM_053922.1	ND		4.4**	1.0
16	Carnitine palmitoyltransferase 1a	Cpt 1a	NM_031559.2	6.5**	0.51	ND	
17	Carnitine palmitoyltransferase 1b	Cpt 1b	NM_013200.1	ND		8.9**	0.95
18	Protein kinase, AMP-activated, alpha 1 catalytic subunit	Prkaa1	NM_019142.2	1.5	0.7	4.6**	1.2
19	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Prkag2	NM_184051.1	ND		3.6	0.5
20	Protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	NM_023991.1	2.8*	0.5	10.9**	1.7
21	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	NM_031976.1	1.2	0.4	ND	
22	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	NM_022627.2	1.6	0.4	ND	
23	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	Prkag1	NM_013010.2	ND		4.6*	1.2
24	Mitofusin 2 (Mfn2), mRNA	Mfn2	NM_130894.4	3.4*	0.51	2.9	0.41
25	Mitofusin 1 (Mfn1), mRNA	Mfn1	NM_138976.1	4.1**	0.23	2.6**	0.31

Values are expressed as mean ± SD (n=8). Significance between groups * p< 0.05, ** p< 0.01 on applying one way ANOVA followed by Dunnett test.

Table 4: Differentially expressed genes in 4-HIL treated L6 myotubes as compared with untreated group

Fold changes in gene expression of 4-HIL treated L6 myotubes					
S. No.	Gene Name	Gene Bank abbreviation	Accession no.	Fold Change in gene expression of L6 myotubes	
				Mean	±SD
1	Transcription factor A	Tfam	NM_031326.1	2.2	0.64
2	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1a	NM_031347.1	3.8*	0.52
3	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	Ppargc1b	NM_176075.2	2.8*	0.35
4	Peroxisome proliferator activated receptor gamma	Pparg	NM_013124.3	0.3	0.03
5	Peroxisome proliferator activated receptor alpha	PPAR α	NM_013196.1	1.3	0.15
6	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	Atp5f1	NM_134365.2	2.3	0.12
7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	Ndufa10	NM_199495.2	3.5**	0.07
8	Succinate dehydrogenase complex, subunit A, flavoprotein	Sdha	NM_130428.1	1.8	0.64
9	Cytochrome c oxidase	Cox6b1	NG_028330.1	4.3**	0.07
10	Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	NM_012751.1	2.1	0.02
11	Solute carrier family 2 (facilitated glucose transporter), member 3	Slc2a3	NM_017102.2	3.1*	0.06
12	Glucose transporter-3	GLUT3	U17978.1	1.1**	0.12
13	Nuclear respiratory factor 1	Nrf1	NM_001100708 .1	2.2	0.41
14	Acetyl-CoA carboxylase alpha	Acaca	NM_022193.1	4.1**	1.31
15	Acetyl-CoA carboxylase beta	Acacb	NM_053922.1	1.3	0.21
16	Carnitine palmitoyltransferase 1a	Cpt 1a	NM_031559.2		
17	Carnitine palmitoyltransferase 1b	Cpt 1b	NM_013200.1	3.9*	0.62
18	Protein kinase, AMP-activated, alpha 1 catalytic subunit	Prkaa1	NM_019142.2	1.6	0.32
19	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Prkag2	NM_184051.1	2.5**	0.75
20	Protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	NM_023991.1	4.4**	0.81
21	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	NM_031976.1	2.7*	0.41
22	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	NM_022627.2	2.2	0.72
23	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	Prkag1	NM_013010.2	1.1	0.16
24	Mitofusin 2	Mfn2	NM_130894.4	3.2**	0.03
25	Mitofusin 1	Mfn1	NM_138976.1	2.9*	0.24

Values are expressed as mean ± SD (n=3). Significance between groups * p< 0.05, ** p< 0.01 on applying one way ANOVA followed by Dunnett test.

3.5. 4HIL treatment selectively up-regulated the genes involved in mitochondrial biogenesis

4-HIL causes significant effect on gene expression profile of liver, skeletal muscles of treated diabetic rats as well as L6 myotubes. Expression of AMPK was increased significantly in both liver and muscle of treated animals and in the L6 myotubes as well as compared to untreated control group. Expression of Ppargc1a, Ppargc1b, an important regulators of mitochondrial biogenesis was increased in both animal and L6 myotubes (Wu, Puigserver, Andersson et al., 1999). Expression of PPARγ and PPARα was decreased in both liver and muscle which suggest that 4-HIL causes decrease in adipogenesis and enhanced catabolic functions in diabetic rats **(Table-3).** Expression of important glucose transporters in cells was found increased along with increased expression of AMPK genes, indicating that glucose utilization is increased and condition of hyperglycaemia is improved in treated animals. Increased expression of glucose transporter in L6 myotubes also confirms the increased glucose uptake in response to 4-HIL treatment **(Table -4).**

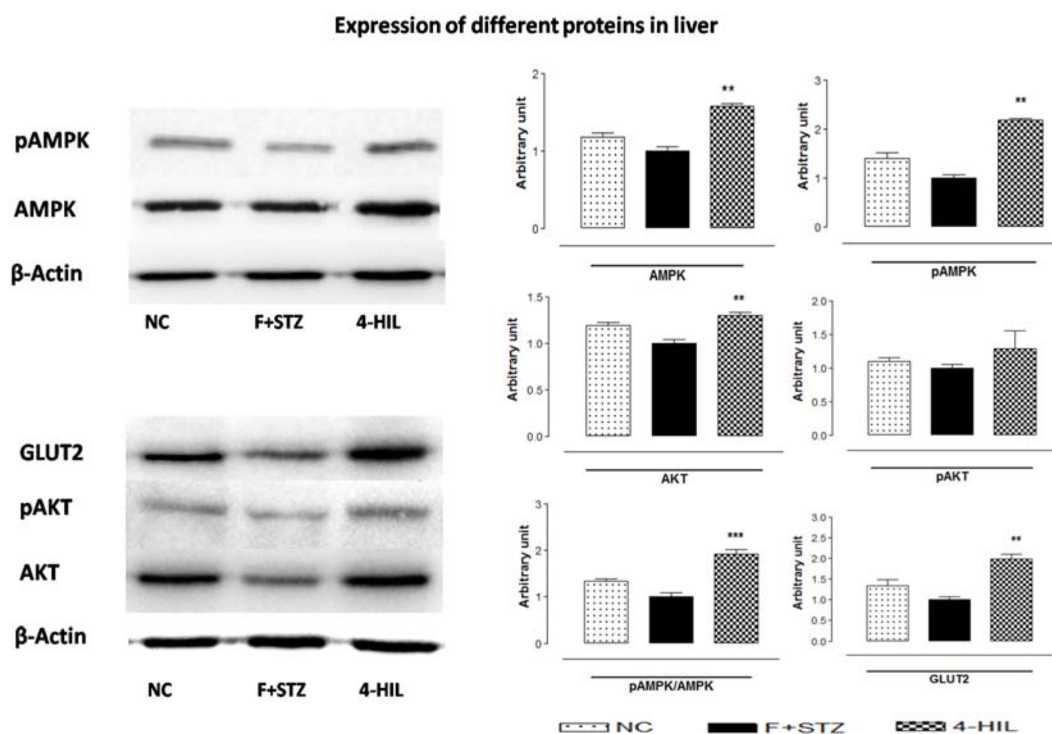

Fig.5. Fold change in protein expression in Liver of 4-HIL treated High fructose diet fed low dose STZ induced rats (n=8). High Fructose diet and streptozotocin induced diabetic control (F+STZ), 4-

Hydroxyisoleucin (4-HIL), Normal control (NC). Values are expressed as mean ± SD. Significance between groups * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ on applying one way ANOVA followed by Dunnett test.

3.6. Expression of AMPK and its phosphorylation increased on 4HIL treatment

Immunoblotting studies confirmed that expression of AMPK and pAMPK was found increased in both muscle and liver. Although we did not found significant increase in liver **(Fig. 5)** of treated animal but in muscle change was significant. Expression of GLUT 4 was increased in muscle, next we found that expression of AKT and pAKT, a downstream regulator of insulin signalling pathway was increased suggesting that glucose metabolism was improved in treated animals **(Fig. 6).** We further investigate effect of 4-HIL treatment on L6 myotubes according to the experimental design and found that expression of both AMPK and pAMPK was decreased when treated with compound C. Expression of GLUT4, AKT and pAKT was also decreased as well. When treated the cells with compound C along with 4-HIL we found that expression of AMPK and pAMPK was improved with that expression of GLUT4, AKT and pAKT was also restored **(Fig. 7).** Treatment with 4-HIL alone causes increased expression of AMPK and pAMPK as well as GLUT4, AKT and pAKT by many folds.

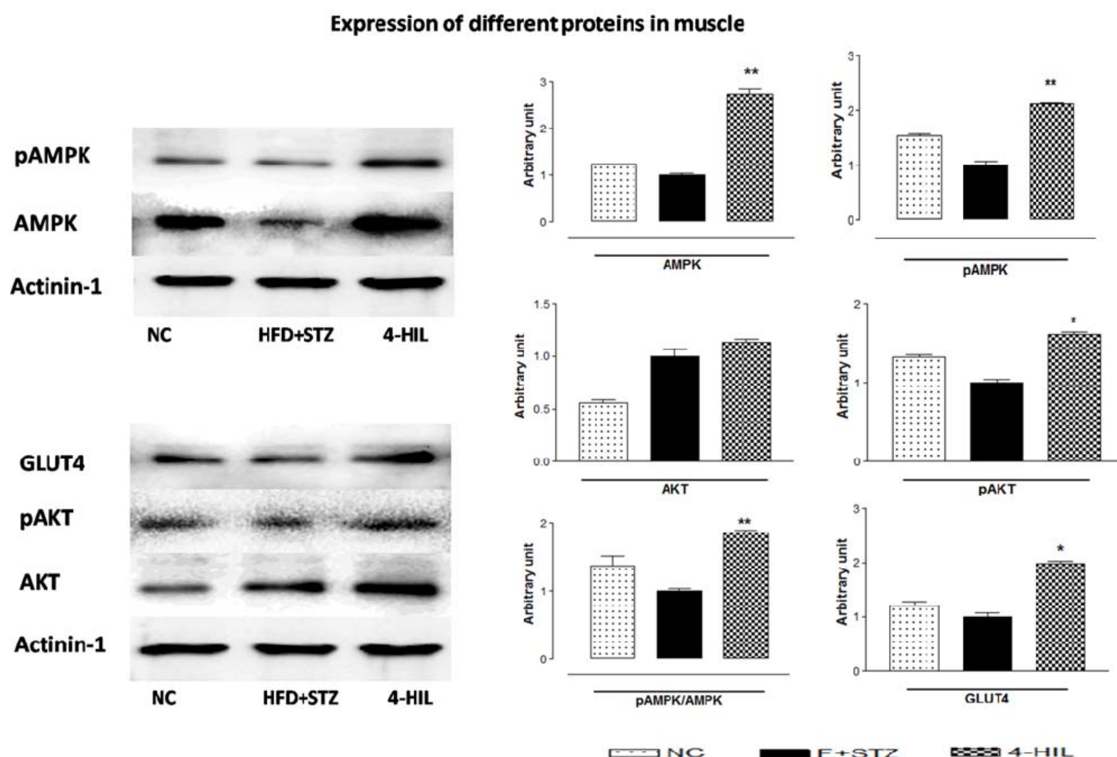

Fig.6. Fold change in protein expression in Muscle of 4-HIL treated High fructose diet fed low dose STZ induced rats (n=8). High Fructose diet and streptozotocin induced diabetic control (F+STZ), 4-

Hydroxyisoleucin (4-HIL), Normal control (NC). Values are expressed as mean ± SD. Significance between groups * p< 0.05, ** p< 0.01 on applying one way ANOVA followed by Dunnett test.

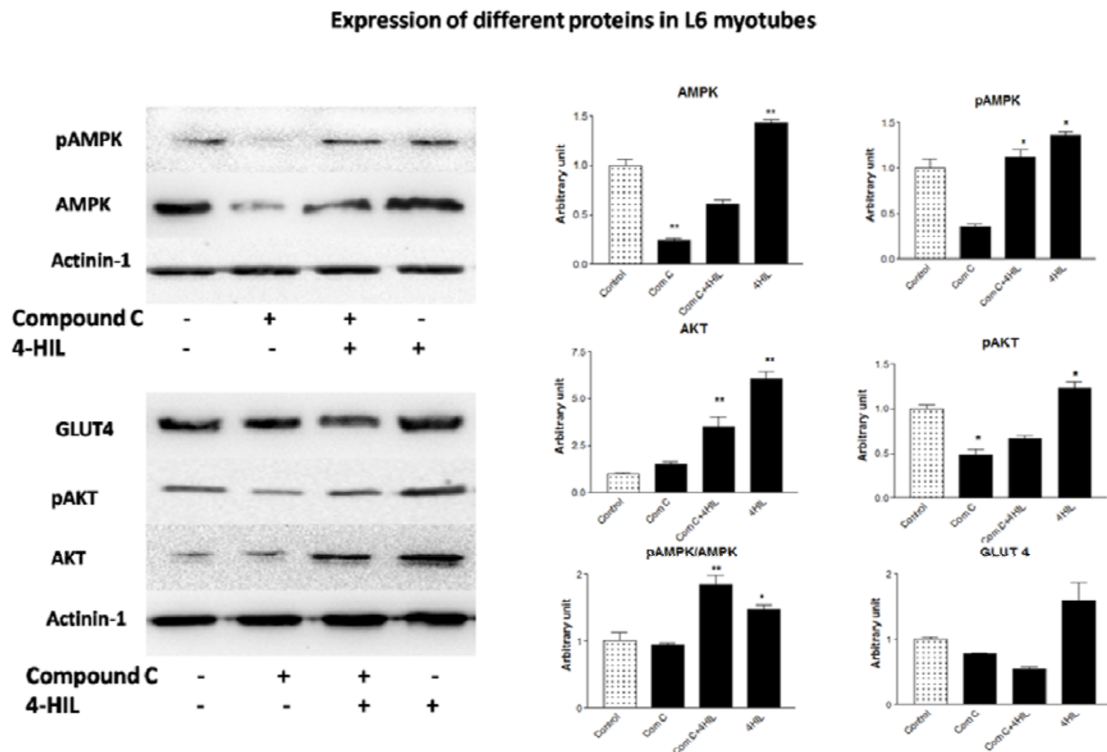

Fig.7. Fold change in protein expression of L6 myotubes, differently treated with 4-HIL and compound C (n=3). Values are expressed as mean ± SD. Significance between groups * p< 0.05, ** p< 0.01 on applying one way ANOVA followed by Dunnett test.

4. Discussion:

High fructose diet feeding causes accumulation of lipids in body which in turn decreases the insulin sensitivity and develops hyperglycaemia (Storlien, Oakes, Pan et al., 1993,Lee, Miles, Khoursheed et al., 1994). This study shows the effect of 4-HIL on high fructose diet fed STZ induced diabetic rats. 4-HIL cause improvement in glucose tolerance and improves insulin sensitivity of diabetic rats. In addition to this, it causes improvement in lipid profile as well as serum insulin level of treated diabetic rats. In support to this antidiabetic as well as antidyslipidemic activities of 4-HIL have been reported in some previous studies (Sauvaire et al., 1998,Broca, Breil, Cruciani-Guglielmacci et al., 2004).

AMPK plays key role in cellular energy homeostasis (Carling, 2004). Insulin mediated glucose uptake is due to activation of AMPK, which in-turn activate the AKT thus act on insulin signalling pathway (Ji, Zhang, Liu et al., 2013). In Liver Gluconeogenesis is suppressed by the activation of AMPK. There are studies reported which shows with

decrease in AMPK expression hyperglycaemia and insulin resistance develop (Chen, Wang, Sun et al., 2010). In high fructose diet fed diabetic rats we also observed expression level changes in AMPK and AKT. We therefore hypothesized that hypoglycaemic properties shown by 4-HIL in high fructose diet fed diabetic rats may be due to the alteration in expression of AMPK, AKT and their phosphorylated forms. It may have important role in increase of glucose uptake in response to AMPK activation (Fisher, Gao, Han et al., 2002,Jakobsen, Hardie, Morrice et al., 2001). L6 cell were incubated with compound C, which resulted in, inhibition of AMPK and subsequently decrease in glucose uptake and when 4-HIL along with compound C was incubated the glucose uptake restored the L6 myotubes which confirms our hypothesis.

In Gene expression profile analysis we found that along with AMPK expression of PGC-1α, PGC-1β and NRFs was increased in 4-HIL treated animals as well as in L6 myotubes. These results are in agreement with the previous studies which shows activation of AMPK results in to increase in expression of PGC-1α (Jäger, Handschin, Pierre et al., 2007). In addition to PGC-1α, expression of Tfam, mitofusin 1, mitofusin 2 was also found to be up-regulated in both the tissues. Tfam is a transcription factor plays role in mitochondrial biogenesis whereas mitofusin 1 and 2 are responsible for mitochondrial fusion (Santel and Fuller, 2001). Expression of some other important mitochondrial proteins such as ATPase, NADH dehydrogenase, succinate dehydrogease and cytochrome c oxidase was also found to be increased in liver and muscle of treated animals. Expression of CPT 1a and 1b (Carnitine palmitoyltransferase1a) was found to be increased in liver and muscles respectively, which is responsible for the translocation of lipids through mitochondrial membranes (Lee, Kerner and Hoppel, 2011).

Immunoblotting studies showed that expression of both AMPK and pAMPK was found to be increased in 4-HIL treated animals as well as L6myotubes. Importantly expression of AKT and pAKT and GLUT4 was also found increased in response to 4-HIL treatment. These results support the findings of gene expression profile studies. Increase in glucose uptake in L6 myotubes is due to increased expression of these molecular mediators. Thus 4-HIL causes activation of AMPK which in-turn causes increase in mitochondrial biogenesis through PGC-1α and NRFs and also it modulates the insulin signalling pathway via AKT (Winder, Holmes, Rubink et al., 2000).

5. Conclusion

In conclusion 4-HIL isolated from the seeds of *T. Foenumgraecum* improves the glucose tolerance and insulin resistance of high fructose diet fed low dose streptozotocin induced diabetic rats. Our mechanistic studies indicated that 4-HIL increases expression of AMPK and Akt along with their phosphorylated forms in liver and mussle as well as L6 myotubes. It also increased the expression of the genes responsible for the mitochondrial biogenesis i.e., PGC-1α and PGC-1β and lipid metabolism was found to be up-regulated in treated animals. All together these result suggest that 4-HIL improves hyperglycaemia and lipid metabolism by mitochondrial biogenesis in diabetic rats via stimulation of AMPK and Akt axis.

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References:

[1] Alberti, K.G.M.M. and Zimmet, P., 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation, Diabetic medicine. 15, 539-553.

[2] Tuomilehto, J., Lindström, J., Eriksson, J.G., Valle, T.T., Hämäläinen, H., Ilanne-Parikka, P., Keinänen-Kiukaanniemi, S., Laakso, M., Louheranta, A. and Rastas, M., 2001. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance, New England Journal of Medicine. 344, 1343-1350.

[3] Kelley, D.E., Mokan, M., Simoneau, J.-A. and Mandarino, L., 1993. Interaction between glucose and free fatty acid metabolism in human skeletal muscle, Journal of Clinical Investigation. 92, 91.

[4] Bezerra, R.M., Ueno, M., Silva, M.S., Tavares, D.Q., Carvalho, C.R., Saad, M.J. and Gontijo, J.A., 2001. A high-fructose diet induces insulin resistance but not blood pressure changes in normotensive rats, Braz J Med Biol Res. 34, 1155-60.

[5] Zavaroni, I., Sander, S., Scott, S. and Reaven, G.M., 1980. Effect of fructose feeding on insulin secretion and insulin action in the rat, Metabolism. 29, 970-973.

[6] Stark, A., Timar, B. and Madar, Z., 2000. Adaption of Sprague Dawley rats to long-term feeding of high fat of high fructose diets, European journal of nutrition. 39, 229-234.

[7] Smyth, S. and Heron, A., 2006. Diabetes and obesity: the twin epidemics, Nature medicine. 12, 75-80.

[8] Koehn, F.E. and Carter, G.T., 2005. The evolving role of natural products in drug discovery, Nat Rev Drug Discov. 4, 206-20.

[9] Bailey, C.J. and Day, C., 1989. Traditional plant medicines as treatments for diabetes, Diabetes care. 12, 553-564.

[10] Ocvirk, S., Kistler, M., Khan, S., Talukder, S.H. and Hauner, H., 2013. Traditional medicinal plants used for the treatment of diabetes in rural and urban areas of Dhaka, Bangladesh--an ethnobotanical survey, Journal of ethnobiology and ethnomedicine. 9, 43.

[11] Sharma, R. and Raghuram, T., 1990. Hypoglycaemic effect of fenugreek seeds in non-insulin dependent diabetic subjects, Nutrition research. 10, 731-739.

[12] El-Soud, N.H.A., Khalil, M., Hussein, J., Oraby, F. and Farrag, A.H., 2007. Antidiabetic effects of fenugreek alkaliod extract in streptozotocin induced hyperglycemic rats, J Appl Sci Res. 3, 1073-1083.

[13] Zia, T., Hasnain, S.N. and Hasan, S., 2001. Evaluation of the oral hypoglycaemic effect ofTrigonella foenum-graecum L.(methi) in normal mice, Journal of ethnopharmacology. 75, 191-195.

[14] Al-Habori, M. and Raman, A., 1998. Antidiabetic and hypocholesterolaemic effects of fenugreek, Phytotherapy research. 12, 233-242.

[15] Sauvaire, Y., Petit, P., Broca, C., Manteghetti, M., Baissac, Y., Fernandez-Alvarez, J., Gross, R., Roye, M., Leconte, A., Gomis, R. and Ribes, G., 1998. 4-Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion, Diabetes. 47, 206-10.

[16] Broca, C., Gross, R., Petit, P., Sauvaire, Y., Manteghetti, M., Tournier, M., Masiello, P., Gomis, R. and Ribes, G., 1999. 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties, Am J Physiol. 277, E617-23.

[17] Jaiswal, N., Maurya, C.K., Venkateswarlu, K., Sukanya, P., Srivastava, A.K., Narender, T. and Tamrakar, A.K., 2012. 4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway, European journal of nutrition. 51, 893-898.

[18] Fowden, L., Pratt, H.M. and Smith, A., 1973. 4-Hydroxyisoleucine from seed of Trigonella foenum-graecum, Phytochemistry. 12, 1707-1711.

[19] Alcock, N.W., Crout, D.H., Gregorio, M.V., Lee, E., Pike, G. and Samuel, C.J., 1989. Stereochemistry of the 4-hydroxyisoleucine from Trigonella foenum-graecum, Phytochemistry. 28, 1835-1841.

[20] Bezerra, R.M.N., Ueno, M., Silva, M.S., Tavares, D.Q., Carvalho, C.R.O. and Saad, M.J.A., 2000. A High Fructose Diet Affects the Early Steps of Insulin Action in Muscle and Liver of Rats, The Journal of nutrition. 130, 1531-1535.

[21] Sawada, K., Kawabata, K., Yamashita, T., Kawasaki, K., Yamamoto, N. and Ashida, H., 2012. Ameliorative effects of polyunsaturated fatty acids against palmitic acid-induced insulin resistance in L6 skeletal muscle cells, Lipids Health Dis. 11, 36-44.

[22] Jaiswal, N., Maurya, C.K., Venkateswarlu, K., Sukanya, P., Srivastava, A.K., Narender, T. and Tamrakar, A.K., 2012. 4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway, Eur J Nutr. 51, 893-8.

[23] Kwon, S., Kim, Y.J. and Kim, M.K., 2008. Effect of fructose or sucrose feeding with different levels on oral glucose tolerance test in normal and type 2 diabetic rats, Nutr Res Pract. 2, 252-8.

[24] Weksler-Zangen, S., Raz, I., Lenzen, S., Jörns, A., Ehrenfeld, S., Amir, G., Oprescu, A., Yagil, Y., Yagil, C. and Zangen, D.H., 2008. Impaired glucose-stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat, Diabetes. 57, 279-287.

[25] Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods. 25, 402-8.

[26] Li, Q.-X., Xiong, Z.-Y., Hu, B.-P., Zhang, H.-F., Gou, W.-Y., Wang, H.-C. and Gao, F., 2009. Aging-associated insulin resistance predisposes to hypertension and its reversal by exercise: the role of vascular vasorelaxation to insulin, Basic research in cardiology. 104, 269-284.

[27] Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B. and Scarpulla, R.C., 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1, Cell. 98, 115-124.

[28] Storlien, L.H., Oakes, N.D., Pan, D.A., Kusunoki, M. and Jenkins, A.B., 1993. Syndromes of insulin resistance in the rat: inducement by diet and amelioration with benfluorex, Diabetes. 42, 457-462.

[29] Lee, M.-K., Miles, P.D., Khoursheed, M., Gao, K.-M., Moossa, A. and Olefsky, J.M., 1994. Metabolic effects of troglitazone on fructose-induced insulin resistance in the rat, Diabetes. 43, 1435-1439.

[30] Broca, C., Breil, V., Cruciani-Guglielmacci, C., Manteghetti, M., Rouault, C., Derouet, M., Rizkalla, S., Pau, B., Petit, P. and Ribes, G., 2004. Insulinotropic agent ID-1101 (4-hydroxyisoleucine) activates insulin signaling in rat, American Journal of Physiology-Endocrinology And Metabolism. 287, E463-E471.

[31] Carling, D., 2004. The AMP-activated protein kinase cascade–a unifying system for energy control, Trends in biochemical sciences. 29, 18-24.

[32] Ji, L., Zhang, X., Liu, W., Huang, Q., Yang, W., Fu, F., Ma, H., Su, H., Wang, H. and Wang, J., 2013. AMPK-Regulated and Akt-Dependent Enhancement of Glucose Uptake Is Essential in Ischemic Preconditioning-Alleviated Reperfusion Injury, PloS one. 8, e69910.

[33] Chen, L., Wang, F., Sun, X., Zhou, J., Gao, L., Jiao, Y., Hou, X., Qin, C. and Zhao, J., 2010. Chronic ethanol feeding impairs AMPK and MEF2 expression and is associated with GLUT4 decrease in rat myocardium, Experimental & molecular medicine. 42, 205-215.

[34] Fisher, J.S., Gao, J., Han, D.-H., Holloszy, J.O. and Nolte, L.A., 2002. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin, American Journal of Physiology-Endocrinology And Metabolism. 282, E18-E23.

[35] Jakobsen, S.N., Hardie, D.G., Morrice, N. and Tornqvist, H.E., 2001. 5′-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside, Journal of Biological Chemistry. 276, 46912-46916.

[36] Jäger, S., Handschin, C., Pierre, J.S.-. and Spiegelman, B.M., 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α, Proceedings of the National Academy of Sciences. 104, 12017-12022.

[37] Santel, A. and Fuller, M.T., 2001. Control of mitochondrial morphology by a human mitofusin, Journal of cell science. 114, 867-874.

[38] Lee, K., Kerner, J. and Hoppel, C.L., 2011. Mitochondrial carnitine palmitoyltransferase 1a (CPT1a) is part of an outer membrane fatty acid transfer complex, Journal of Biological Chemistry. 286, 25655-25662.

[39] Winder, W., Holmes, B., Rubink, D., Jensen, E., Chen, M. and Holloszy, J., 2000. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle, Journal of applied physiology. 88, 2219-2226.