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Orginal Research Article

Activating the AMPK by DHPO to Mitigate Lipid Abnormalities and Insulin Resistance

Aboajela Ramadan Imbark Ajaj[1], Ashraf Mohamed Albakoush[2], Azab Elsayed Azab[3,*]
[1]Department of Anesthesia, Faculty of Medical Technology, Surman, Sabratha University, Libya
[2]Department of Medical Laboratory Technology, Faculty of Medical Technology, Surman, Sabratha University, Libya
[3]Department of Zoology, Faculty of Science, Alejelat, Zawia University, Libya

***Corresponding Author:**
Azab Elsayed Azab
Email: azabelsaied@yahoo.com

Abstract: Metabolic syndrome such as type-2 diabetes and obesity is becoming formidable health issue around the world. This study demonstrate the effect of a small molecule 2-(3,4-dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate (DHPO), on metabolic syndrome diet induce obese rats(8-9 weeks old) . Rats were divided into three groups, Two groups were fed either a corn starch–rich (C) or high-carbohydrate, high-fat (H) diet for 16 weeks, the third group (HD) was fed high-carbohydrate, high-fat diet, for the first 8 weeks and the diet was supplemented with DHPO (0.4 g/kg food) for a additional 8 weeks. H and C diets contained 68% carbohydrates, as fructose and sucrose in H diet and as polysaccharides in C diet, and C diet contained 24 and 0.7% fat. The high-carbohydrate, high-fat diet produced obesity, hypertension, dyslipidaemia, impaired glucose tolerance, NAFLD, cardiovascular remodelling, and endothelial dysfunction. DHPO promote glucose disposal and corrected dyslipidaemia in dietary rats (high-carbohydrate, high-fat) by enhanced insulin signalling pathway such as AMPK. In addition, DHPO augmented glucose-uptake in gastrocnemius muscles. Therefore, DHPO may be the novel component that improve endothelial dysfunction and impaired glucose tolerance which cause type-2 diabetes**.**

Keywords: AMPK, DHPO, lipid abnormalities, insulin resistance

INTRODUCTION

Metabolic syndromes which include insulin resistance, dyslipidaemia, impaired glucose tolerance, central obesity and elevated blood pressure still the common public health issue. All these disturbances lead to increase the incidence of cardiovascular disease, non-alcoholic fatty liver disease, kidney dysfunction and type 2diabetes [1]. Insulin resistance and impaired glucose tolerance generate type 2 diabetes. There are two types of diabetes mellitus, type 1 occurs by autoimmune destruction of beta cells in pancreas that are responsible for insulin production. It is treated just by insulin either via insulin subcutaneous injection or pump. Type 2 diabetes is non-insulin dependent, it caused by metabolic disorder [1]. In type 2 diabetes, there is no imperfection in pancreas and insulin is find in blood normally, but the defect in insulin receptors (alpha, beta) lose the sensitivity to insulin which activate the cell to take glucose inside the cell that play significant role in generating energy in the cell. The main cause of type 2 diabetes is lifestyle factor such as insufficient physical activity, unhealthy eating (high carbohydrate& high fat food) which lead to obesity. Also, another reason for type 2 diabetes is a strong genetic predisposition. Type 2 diabetes usually affects old people (people over 40 years old).

DHPO is a novel synthetic small molecule that mitigates lipid abnormalities and insulin resistance which lead to type-2 diabetes and obesity. The chemical structure of DHPO is 2-(3,4-dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate, and it is an orange coloured solid, the empirical formula is $C_{13}H_9NO_2$ and the molecular weight is 211.22. DHPO elevated the cellular uptake of 2-deoxy-D-glucose (2DG) that associated with augment in the phosphorylation of Mitogen-activated protein (MAP) kinase (the- 172) and it keep the same effect in acetyl- CoA carboxylase without any alteration in the phosphorylation of Akt of insulin receptor [2]. Furthermore, DHPO elevated skeletal muscle glucose-uptake by effecting on AMPK signalling pathway which play important role in the carbohydrate metabolism. Thus, DHPO is novel compound that enhances glucose disposal and insulin signalling which refer to the sensitivity of insulin receptor (alpha & beta) to insulin and palliate insulin resistance by activation of the AMPK signalling pathway [2]. The following picture is the chemical structure for DHPO.

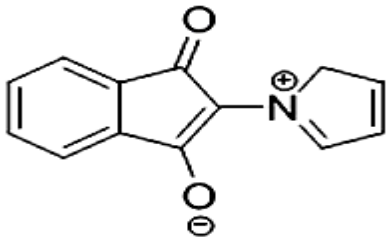

Fig-1: The chemical structure of DHPO

The AMP-activated protein kinase (AMPK) system is the main sensor of cellular energy status that is found in eukaryotic cells. Therefore, it plays significant role in regulation of cellular energy homeostasis. It is activated when cellular ATP supply is depleted and increase in AMP levels in conditions such as low glucose, ischemia, and hypoxia [3]. AMPK exists as a heterotrimeric complex that consists of a catalytic alpha subunit and regulatory beta and gamma subunits. There are two pathways to AMPK as a master regulator of cellular energy AMPK activation positively regulates signalling pathways and AMPK negatively regulates. In positively manner AMPK replenish cellular ATP supply via promoting both the transcription and translocation of GLUT4 which lead to rise in insulin-stimulated glucose uptake. Furthermore, AMPK stimulates catabolic mechanisms; for example, it activates glycolysis and fatty acid oxidation by inhibition of ACC and activation of Phosphofructokinase 2 [4]. AMPK negatively regulates Snare proteins central to ATP consumption mechanisms such as TORC2, glycogen synthase, SREBP-1 and TSC2 that lead to inhibit lipid and protein synthesis and gluconeogenesis [5]. Thus, compound that activate AMPK are considered significant treatment for type 2 diabetes and obesity. The following diagram explains the AMPK Signalling Pathways (Figure 2).

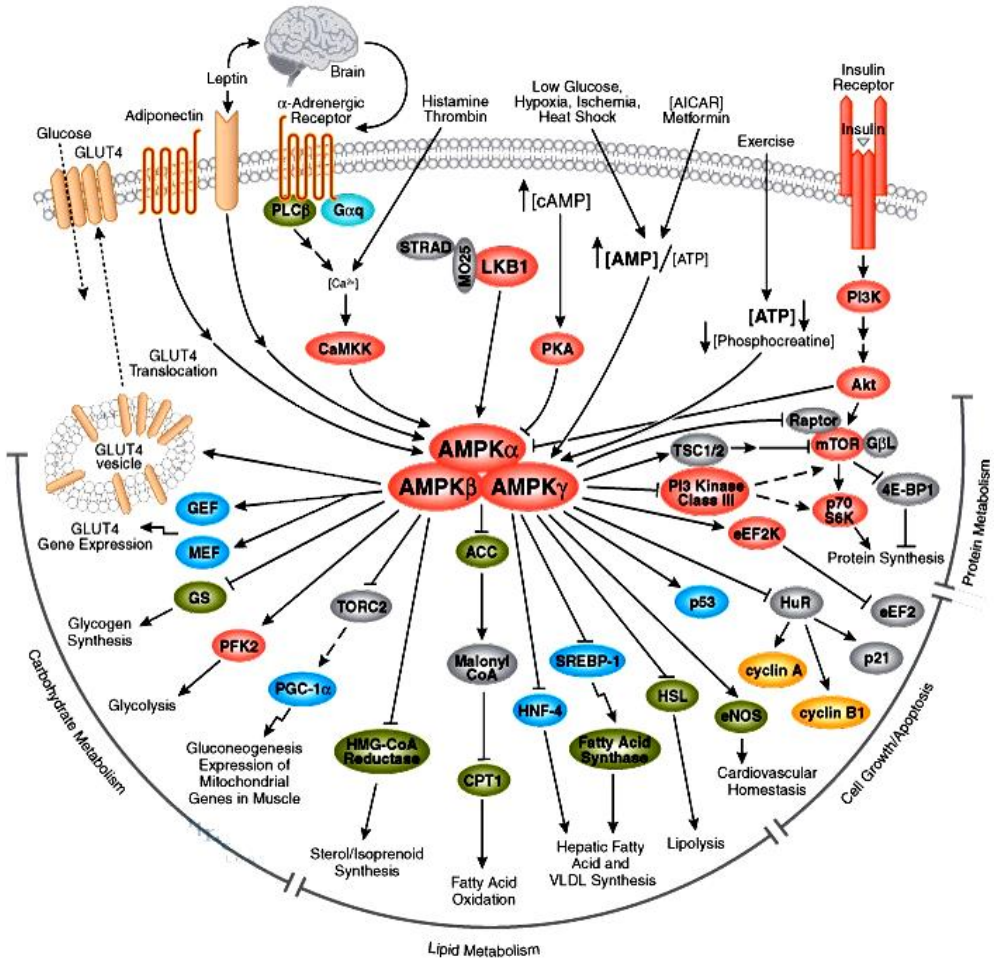

Fig-2: The positive and negative signalling pathway for AMPK (http://www.cellsignal.com/reference/pathway/AMPK.html)

In this study, we detect that DHPO has significantly improved lipid and carbohydrate abnormalities which associate with insulin resistants in rats model of high-carbohydrate and high-fat diet, that generate metabolic syndrome (insulin resistance, dyslipidaemia, impaired glucose tolerance, obesity and high blood pressure). Also, thoracic aorta was isolated to measure vascular reactivity. Furthermore, cardiovascular and liver structure & function were examined

MATERIALS AND METHODS

Diet-induced metabolic syndrome in rats

All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, weighing 336 ± 1 g, n =36) were obtained from the University of Southern Queensland Animal House facility. The rats were randomly divided into three experimental groups and were fed with corn starch diet (C; n =12) high-carbohydrate, high-fat diet (H) diet (n = 12) or high-carbohydrate, high-fat diet+ DHPO (HD; n=12).

The C diet contained 570 g of corn starch, 155 g of powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia; all nutritional parameters of this diet meet or exceed the National Research Council, Canada, guidelines for rats and mice), 25 g of Hubble, Mendel and Wakeman salt mixture, 250 g of water per kilogram of diet. H diet consisted of 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of Hubble, Mendel and Wakeman salt mixture, and 50 g of water per kilogram of diet. HD rats were fed with the H diet for first 8 weeks and then the diet was supplemented with DHPO (0.4g/kg food). In addition, the drinking water for the H and HD groups were supplemented with 25% fructose. Rats were given *ad libitum* access to food and water and were individually housed in temperature-controlled 12-hour light– dark conditions. Energy intake was calculated previously described [6].

Physiological and Metabolic Parameters

Body weight, food and water intakes were measured daily. Oral glucose tolerance tests were performed every fourth week after determining overnight fasting blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters (Abbott Laboratories, Bedford, MA). For overnight fasting, rats were deprived of all types of diets for 12 hours. Fructose-supplemented drinking water in H and HD groups was replaced with normal drinking water for the overnight food deprivation period. Rats were given a glucose load of 2 g/(kg body weight) as 40% glucose solution via oral gavage, and blood glucose concentrations were measured again 30, 60, 90 and 120 minutes after oral glucose administration. Abdominal circumference and body length (nose to anus) were measured every fourth week using a standard measuring tape under light anesthesia with Zoletil [tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal (IP); Virbac, Peakhurst, New South Wales, Australia]. Plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were also measured as in a previous study [7].

Body Composition Measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 16 weeks of feeding (2 days before rats were euthanized for pathophysiological assessments) using a Norland XR36 DXA instrument (Norland Corp, Fort Atkinson, WI). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp). The precision error of lean mass for replicate measurements, with repositioning, was 3.2% [6].

Terminal experiments

Rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg, IP; Virbac). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, New South Wales, Australia) was injected through the right femoral vein. The abdomen was then opened and blood (5 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. Blood was centrifuged at 5,000 × g for 15 minutes to obtain plasma. Plasma was stored at -20^{o}C for further characterization [6]. Hearts were removed from rats for isolated Langendorff preparation, and thoracic aorta was used for vascular reactivity studies. Liver, kidney, heart, spleen, and different fat pads were removed from these rats and weighed. Weights of these organs were normalised relative to the tibial length at the time of removal (expressed as tissue weight in milligram per millimetre tibia length) [7].

Assessment of hepatic function

Livers were isolated after euthanasia and weighed. Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP), were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400) as previously described [6].

Assessment of cardiovascular structure and function

Systolic blood pressure measurements: The systolic blood pressure of rats was measured at the end of feeding period under light sedation with Zoletil by using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments) [6].

Echocardiography: Echocardiographic examination (Phillips iE33, 12-MHz transducer) was performed to assess the cardiovascular structure and function in all the rats. The examination was performed as previously described [8].

Left ventricular function: Isolated Langendorff heart preparations (n = 9) were used to assess left ventricular function of the rats in each group as in previous studies [12]. After performing Langendorff heart perfusion studies, the heart was separated into right ventricle and left ventricle (with septum) for weighing.

Vascular reactivity: Thoracic aortic rings (4 mm in length; n = 10 from each group) were suspended in an organ bath maintained at 35°C and filled with Tyrode physiological salt solution bubbled with 95% O_2-5% CO_2 and allowed to stabilise at a resting tension of 10 mN. Cumulative concentration-response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia) and cumulative concentration-response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) following submaximal (70%) contraction to noradrenaline [6]

STATISTICAL ANALYSIS

Values are presented as mean ± SEM. Differences between the groups were determined by oneway analysis of variance. Statistically significant variables were treated with Neumann–Keuls post hoc test to compare all the groups of animals. $P<0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows.

RESULTS

The results obtained from this experiment is shown as from the values of final body weight, water intake, food intake, energy intake, feed efficiency, BMI, DHPO intake, abdominal circumference, whole-body lean mass, whole-body fat mass, bone mineral density, bone mineral content, basal blood glucose concentration, area under curve, total cholesterol, triglycerides, non-esterified fatty acids (NEFA) and total abdominal fat pads constants for the entire group of rats. First the body weight was elevated in H rats compared with C rats at 16 week while it did not differ between the H and HD rats at 16 week (Table1 & Figure 3A) .

Table 1: Effects of DHPO on physiological and metabolic parameters

Variables	***C***	***H***	***HD***
Initial body weight (g)	336 ± 2	339 ± 1	335 ± 1
Final body weight (g)	414 ± 8^{b}	545 ± 10^{a}	542 ± 13^{a}
Water intake (mL/d)	32.6 ± 2.6^{a}	21.1 ± 0.5^{b}	23.1 ± 1.0^{b}
Food intake (g/d)	31.5 ± 2.1^{a}	25.3 ± 0.8^{b}	26.6 ± 0.5^{b}
Energy intake (kJ/d)	365 ± 13^{b}	435 ± 13^{a}	466 ± 11^{a}
Feed efficiency (kJ/g)	0.16 ± 0.03^{b}	0.35 ± 0.01^{a}	0.36 ± 0.02^{a}
BMI (g/cm^2)	0.66 ± 0.01^{b}	0.82 ± 0.02^{a}	0.80 ± 0.02^{a}
DHPO intake (mg /kg/d)	-	-	19.6 ± 0.3
Abdominal circumference (cm)	18.6 ± 0.3^{b}	21.4 ± 0.3^{a}	21.3 ± 0.4^{a}
Whole-body lean mass (g)	299 ± 7	315 ± 9	315 ± 9
Whole-body fat mass (g)	83 ± 11^{b}	170 ± 10^{a}	158 ± 11^{a}
Bone mineral density (g/cm^2)	0.160 ± 0.002^{b}	0.171 ± 0.002^{a}	0.172 ± 0.003^{a}
Bone mineral content (g)	12.31 ± 0.36^{b}	15.24 ± 0.22^{a}	14.77 ± 0.35^{a}
Basal blood glucose concentration (mmol/L)	3.7 ± 0.2^{b}	5.1 ± 0.2^{a}	4.7 ± 0.2^{a}
Area under curve (mmol/L·min)	581 ± 23^{c}	744 ± 14^{a}	631 ± 12^{b}
Total cholesterol (mmol/L)	1.4 ± 0.1^{b}	1.9 ± 0.1^{a}	2.0 ± 0.1^{a}
Triglycerides (mmol/L)	0.4 ± 0.0^{c}	0.9 ± 0.2^{b}	2.2 ± 0.5^{a}
NEFA (mmol/L)	1.3 ± 0.1^{c}	3.2 ± 0.7^{b}	5.7 ± 0.7^{a}
Retroperitoneal fat pads (mg/mm tibial length)	154 ± 16^{b}	332 ± 31^{a}	351 ± 40^{a}
Epididymal fat pads (mg/mm tibial length)	112 ± 11^{b}	206 ± 16^{a}	228 ± 26^{a}
Omental fat pads (mg/mm tibial length)	96 ± 7^{b}	195 ± 13^{a}	184 ± 17^{a}
Total abdominal fat pads (mg/mm tibial length)	362 ± 33^{b}	723 ± 54^{a}	763 ± 79^{a}

Values are presented as mean ± SEM (n = 8–12). Means without a common superscript letter in a row differ ($P < 0.05$). C, corn starch-rich diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HD, high-carbohydrate, high-fat diet-fed rats treated with DHPO.

Although water intake and food intake were higher in C rats than in H rats, it did not differ between the H rats and HD rats (Table1). Energy intake and feed efficiency were higher in the H rats than in the C rats, whereas it did not differ between H rats and HD rats (Table1). Unlike C rats had lower whereas H rats had higher BMI and abdominal circumference, H rats and HD rats had no different in BMI and abdominal circumference (Table1).

There was no different between groups in whole-body lean mass. The whole-body fat mass was lower in C rats than H rats; however, it did not differ between H rats and HD rats. Even though the bone mineral density and bone mineral content are elevated in H rats and reduced in C rats, there is no different in bone mineral density and bone mineral content between H rats and HD rats. Lower basal blood glucose

concentrations in the C rats compared with H rat were no different in H rats and HD rats (Table 1 & Figure 3B). Area under curve was higher in H rats than C rats, and it was lower in HD rats than H rats (Table1). Total cholesterol was lower in the C rats than in the H rats; nevertheless, H rats did not differ in total cholesterol from HD rats. Triglyceride and NEFA were lower in C rats compared with H rats, but it was higher in HD rats compared with H rats. Retroperitoneal, epididymal, and omental fat were lower in C rats compared with H rats while it did not differ between H rats and HD rats.

Table 2: Effects of DHPO on cardiovascular and hepatic functions

Variables	C	H	HD
Left ventricular internal diameter during diastole (mm)	7.77 ± 0.19	8.22 ± 0.15	7.86 ± 0.20
Left ventricular posterior wall thickness during diastole (mm)	1.96 ± 0.04	1.93 ± 0.03	2.04 ± 0.04
Interventricular septum thickness during diastole (mm)	1.96 ± 0.04	1.98 ± 0.03	2.03 ± 0.03
Left ventricular internal diameter during systole (mm)	4.09 ± 0.23	4.12 ± 0.26	4.20 ± 0.17
Systolic volume (μL)	79 ± 11	82 ± 16	80 ± 10
Relative wall thickness	0.51 ± 0.02	0.48 ± 0.01	0.52 ± 0.02
Fractional shortening (%)	48 ± 2	50 ± 2	47 ± 2
Ejection fraction (%)	85 ± 2	87 ± 2	85 ± 2
Ejection time (msec)	85 ± 3	88 ± 2	91 ± 3
E:A ratio	2.06 ± 0.08^{a}	1.64 ± 0.13^{b}	1.89 ± 0.11^{ab}
Estimated left ventricular mass (g)	1.08 ± 0.04	1.16 ± 0.02	1.15 ± 0.04
Systolic blood pressure (mmHg)	164 ± 8	156 ± 4	164 ± 4
Left ventricular (with septum) wet weight (mg/mm tibial length)	18.6 ± 0.5^{b}	20.6 ± 0.4^{a}	21.3 ± 0.2^{a}
Right ventricular wet weight (mg/mm tibial length)	6.1 ± 1.4	4.3 ± 0.2	4.4 ± 0.2
Liver wet weight (mg/mm tibial length)	254 ± 10^{b}	362 ± 10^{a}	351 ± 13^{a}
Plasma ALT activity (U/L)	28 ± 2^{b}	46 ± 4^{a}	30 ± 1^{b}
Plasma AST activity (U/L)	79 ± 6^{b}	104 ± 7^{a}	66 ± 3^{b}
Plasma ALP activity (U/L)	139 ± 14^{c}	302 ± 22^{a}	238 ± 17^{b}

Values are mean ± SEM (n = 8–10). Means without a common superscript letter in a row differ ($P < 0.05$). C, corn starch-rich diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HD, high-carbohydrate, high-fat diet-fed rats treated with DHPO.

Left ventricular internal diameter during diastole, left ventricular posterior wall thickness during diastole, interventricular septum thickness during diastole, left ventricular internal diameter during systole, and systolic volume did not differ between C rats and H rats also between H rats and HD rats (Table2). Moreover, there was no different between all groups in relative wall thickness, fractional shortening, ejection fraction, and ejection time (Table2). E: A ratio was higher in C rats compared with H rats, whereas it was lower in H rats than HD rats. Estimated left ventricular mass and systolic blood pressure did not differ in whole groups of rats. H rats had higher left ventricular wet weight than C rats while H rats and HD rats had same left ventricular wet weight. However, right ventricular wet weight did not differ between all groups. Liver wet weight was lower in C rats compared with H rats, whereas it did not differ between H rats and HD rats. Plasma ALT activity and plasma AST activity were higher in H rats than C rats as well as it were higher in H rats than in HD rats. Although plasma ALP activity was lower in C rats compared with H rats, it was higher in H rats than HD rats (Table2).

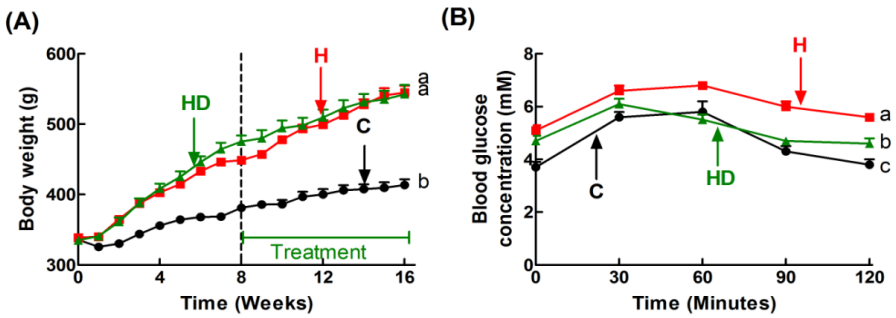

Fig-3: Effects of DHPO on bodyweight (A), and on oral glucose tolerance (B) in C8, H8, and HD12rats. Values are mean ± SEM (n = 8–10). Means without a common superscript letter in a row differ (*P* < 0.05). C, corn starch-rich diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HD, high-carbohydrate, high-fat diet-fed rats treated with DHPO

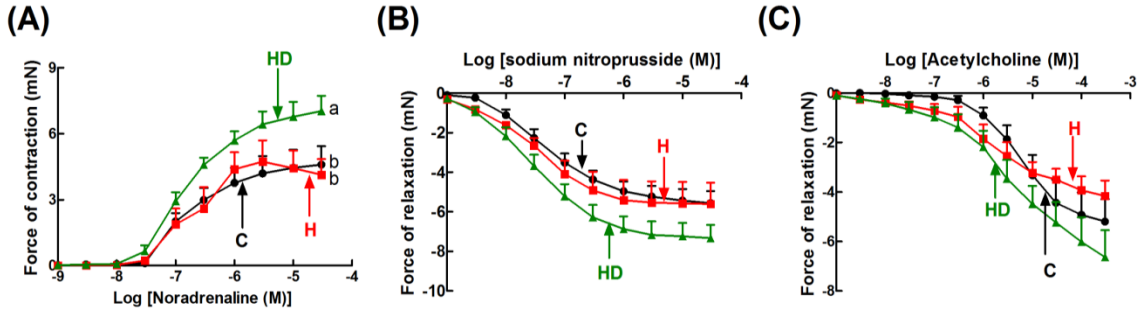

Fig-4: Noradrenaline-induced contraction (A), sodium nitroprusside-induced relaxation (B), and acetylcholine-induced relaxation (C) in thoracic aortic preparations from rats fed C or H diets for 16 weeks, and HD diet for an additional 8 weeks after 8 weeks with H diet

From the organ bath results, the experiment indicates that a graph obtained indicates that in the H rats there is a metabolic syndrome and cardiovascular remodelling due to elevated of fats and carbohydrates which caused hypertension and endothelia dysfunction. The graph of force of contraction (mN) versus Noradrenaline concentration (log M) in H rats and C rats shows that there is a marked decreased contractile coefficient of the vessel due to fibrosis in smooth muscle and endothelia dysfunction whereas the HD rats have marked increased contractile coefficient due to improvement by DHPO (Figure 4A), with increased concentration of noradrenaline. In H rats the graph shown that the force of relaxation (mN) versus acetylcholine concentration (log M) is dramatically declined because fibrosis in endothelial layer of vessels while in HD rats the relaxation abilities are still finding, further relaxation ability in C rats is higher than H rats (Figure 4C). This is also seen in the graph of force of relaxation (mN) versus concentration of sodium nitroprusside (log M), whereby there is a similar trend of decreased relaxation abilities of the blood vessels in the H rats and C rats whereas in HD rats the relaxation abilities are improved (Figure 4B). The relaxation abilities response in blood vessels to sodium nitroprusside indicated more than relaxation abilities response to acetylchline. Due to sodium nitroprussid is endothelial independent, while acetylchline is endothelial dependent.

DISCUSSION

The major purpose of this study is that examined the effects of DHPO on attenuate glucose intolerance in dietary (HCHF) rats' models of insulin resistance by phosphorylation-AMPK. The results obtained from this experiment is shown that the glucose intolerance and endothelial dysfunction in dietary (HCHF) are improved by DHPO. Due to the DHPO stimulate glucose-uptake are by the effect of DHPO on key cellular mediators of insulin signalling which are Akt, adenosine monophosphate-activated protein kinase (AMPK), and acetyl co-A-carboxylase (ACC). Previous study has shown that DHPO promote phosphorylation of Akt, AMPK, and ACC [9]. When DHPO phosphorylate Akt which play important role in insulin receptor signaling pathway the insulin receptor (alpha and beta) become phosphorylation [10]. In addition, this study determined that DHPO induce phosphorylation of AMPK which has significant role on regulate cellular energy by positive and negative pathway. AMPK promotes energy conservation and survival by shutting down anabolism and activating catabolic pathways. The insulin receptor provide a direct link between AMPK and the insulin signalling pathway when AMPK phosphorylates and activates, this pathway promotes energy conservation and survival of muscle exposed to severe glucose deprivation [11]. However, DHPO did not reduce obesity, so may not be effective against obesity or may need to increase dose of DHPO.

CONCLUSION

The High-carbohydrate, High-fat diet-fed Wistar rat model is a typical example of metabolic syndrome which occurs in the human beings with obesity and diabetes mellitus type 2. We can give a conclusion that there is a combination effort by cellular mediators of insulin signalling pathway such as AMPK and Akt that enhances the glucose-uptake in skeletal muscle. Therefore, DHPO can be very significant in the fact that, the research can be utilised as opportunities to develop new noble therapeutic agents for metabolic syndrome such as diabetes mellitus type 2, and this is a major task in clinical medicine management of aged populations in our society.

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