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Ketogenic diet and beta-hydroxybutyrate modulate the hepatic expression of AKT/PI3K/mTOR pathway during treatment of obesity in rats.

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ABSTRACT:

The ketogenic diet (KD) is a dietary plan enriched in fat and lower in carbohydrates designed to treat obesity through boost the production and consumption of ketone bodies. The aim of this study was to explore the hepatic effects of ketogenic diet during treatment of obesity in HFD-rats and to compare these effects with orlistat and β-hydroxybutyrate (BOHB). The rats were assigned into two essential groups: Group I, healthy control group, and Group II: obese rats subdivided into 5 groups: group IIA: untreated obese rats, Group IIB: treated orally with orlistat, Group IIC: treated orally with BOHB, Group IID: treated orally with combination of BOHB and orlistat and Group IIE: feed with KD. After two months of treatments, rats were sacrificed and blood samples and hepatic tissues were obtained for assessments of serum biochemical parameters and BOHB, and hepatic phosphatidylinositol 3-kinases (PI3k), protein kinase B (AKT), mammalian target of rapamycin complex 1 (mTORC1), and sterol regulatory element binding proteins 1c (SREBP-1c) expression at mRNA and protein levels. Only the KD significantly declined the weight gain while all treatments significantly corrected hyperglycemia, elevated insulin levels, and insulin resistance. KD reversed the dysregulated hepatic expression of PI3K, AKT, mTORC1, and SREBP-1c at both mRNA and protein levels. Also, BOHB alone or combined with orlistat resulted in a considerable improvement of the altered genes. The current study's findings provide evidences for the anti-obesity potential of KD and BOHB through the amelioration of glucose and lipid homeostasis, insulin sensitivity and hepatic PI3K /AKT/mTOR pathway.

Keywords: Obesity, ketogenic diet, phosphatidylinositol 3-kinases, mammalian target of rapamycin complex 1, and sterol regulatory element binding proteins 1c.

1. INTRODUCTION

Diabetes Obesity is a complicated metabolic disease, which is brought on by an imbalance between energy consumption and expenditure. Depending on kind and amount of food consumed, as well as lifestyle, this imbalance may have genetic or behavioral origins. The hormonal imbalance of adiponectin and insulin is frequently linked to obesity.Diabetes and cardiovascular disease risks are increased by obesity and losing weight has been proven to ameliorate these conditions **(Andersen & Fernandez, 2013).** According to data from the World Health Organisation (WHO), the prevalence of obesity has tripled globally since 1975. It is estimated that 1.9 billion people (39%) globally are overweight, while 650 million people (13%) are obese **(Di Rosa et al., 2020).** The main risk factors for the development of non-alcoholic fatty liver disorders (NAFLD) include metabolic syndrome, cardiovascular disease, and insulin resistance, all of which are directly caused by obesity **(Uthayakumar & Kotalawala, 2021).** Liver is considered as one of the main organs that affected by obesity as the increased fat intake and elevated circulating fatty acids results in hepatic insulin resistance and ectopic lipid accumulation in liver **(Bugianesi et al., 2005).** Insulin receptor substrate (IRS), phosphatidylinositol-3-kinases (PI3K), and protein kinase B (AKT) are among the proteins and molecules that are activated to carry out the hepatic insulin signaling pathway. Insulin receptor substrates 1 and 2 (IRS-1 and 2) undergo tyrosine phosphorylation when insulin binds to its receptor, initiating this process. **(Previs et al., 2000)**. The IRS triggers PI3K to generate PIP3, which in turn triggers AKT **(Sale & Sale, 2008)**. This leads to the insulin-stimulated uptake of circulating glucose via glucose transporter 4 (GLUT4), increased protein synthesis via mTORC1 activation, and increased lipogenesis via SREBP-1C activation. Thus, changes in any of them along the pathway result in insulin resistance, which in turn causes the development of NAFLD and obesity (Polyzos et al., 2019; Uthayakumar & Kotalawala, 2021).

Pharmacological and/or physiological approaches can be used to treat obesity;

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however, patient noncompliance with the physiological method makes it ineffective. The pharmacological approach employs anti-obesity or weight-loss medications to reduce or manage weight by altering appetite or calorie absorption; orlistat, for instance, works as an inhibitor of the lipase enzyme to prevent the digestion and absorption of triglycerides. Patients who are chronically obese and require months to lose weight find these therapies useless because they are only recommended for short-term use. Therefore, scientists are searching for novel ways to address obesity and the health concerns that go along with it (Chen et al., 2020; Alsenousy et al., 2022; Oriquat et al., 2023).

One of the effective treatments of obesity that widely used is the ketogenic diets (KD). The ketogenic diet (KD), or low carbohydrates-high fat diet, replicates a "fasting state" leading to enhanced fatty acid oxidation and induction of ketoacidosis or production of ketone bodies namely beta-hydroxybutyric acid (BOHB) and acetoacetic acid (Paoli, 2014). When glucose isn't available, the brain uses ketones, which are made from fatty acids in the liver and act as a substitute energy source for glucose (Walczyk & Wick, 2017). Despite being an effective tool in the short- to medium-term fight against obesity (Bueno et al., 2013), KDs raise some concerns among physicians (Freedman et al., 2001), (Paoli et al., 2013). Many of the concerns may be related to a general lack of understanding regarding the physiological mechanisms at play and possible detrimental metabolic consequences on various organs, such as the liver and vascular system.

The present study aimed to explore the hepatic effects of KD during treatment of obesity in rats and to compare its effects with the orlistat as a conventional drug of obesity. Furthermore, the anti-obesity potential of BOHB alone or combined with orlistat was explored.

2. MATERIALS AND METHODS

2.1. Constituents of the diets

The control, obesogenic and ketogenic diet were presented **in Table 1** as follow**:**

Table 1: Constituents of the diets in this study.

Macronutrients (g/kg diet)	Control diet (Kamel et al., 2014)	Obesogenic diet (Kamel et al., 2014)	Ketogenic diet (Abdelsalam et al., 2023)
Protein			
• **Casein**	**220**	**144**	**150**
Carbohydrates			
• **Corn Starch**	**631**	**150**	**50**
• **Dextrose**		**163**	
• **Sucrose**		**280**	
Fat			
• **Lard**	**-**	**148**	**700**
• **corn oil**	**43**	**20**	**-**
Cellulose	**54**	**50**	**50**
Vitamin mix	**10**	**10**	**10**
Mineral mix	**40**	**35**	**40**
Total energy (kcal/g diet)	**3.8**	**4.5**	**7.1**

2.2. Experimental animals

48 Wistar albino male rats, 2 months old, weighing between 80 and 90 grammes, were used. The rats were acquired from the animal house of Medical Research Institute, Alexandria University, Egypt. Prior to experimentation, rats were housed in standard cages in a well-ventilated environment (25 ± 2 °C, 43 ± 3 relative humidity), with free access to food and water and a 12-hour light/dark cycle

2.3. Ethical statement

The Institutional Animal Care and Use Committee (IACUC) of Alexandria University in Egypt granted approval for all experiments, which pursued the standards in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) (Approval No.: AU01219101613). The National Research Council's guide for the use and care of laboratory animals, as well as ARRIVE guidelines, were followed by the study.

2.4. Obesity induction

After two weeks of acclimatization, rats were induced with obesity by feeding them an obesogenic diet for three months. as mentioned in **Table 1**. Rats which became at least 20% heavier than the mean weight of control rats with the same age were considered obese and used for completion the study.

2.5. Experimental design

48 Wistar albino male rats were divided into two main groups: healthy control group that consists of 8 healthy male rats and obese group that consists of 40 obese male rats that were randomly subdivided into five groups (8 rats each) according to the treatment: Untreated-obese group, that were fed obesogenic diet **(Kamel et al., 2014)**, Orlistat-treated obese group, that were orally treated with orlistat (OrlyR from EVA PHARMA , Egypt, Product Code: 11659) dissolved in dimethyl sulfoxide at a dose of 30 mg/kg daily **(Gomaa et al., 2019).** BOHB-treated obese group, that were orally treated with BOHB at dose of 200 mg/kg daily

(Caminhotto et al., 2017), combined-treated obese group, that were treated with a combination of oral BOHB and orlistat daily with the same doses, KD-treated obese group, that were feed KD **(Abdelsalam et al., 2023)**. All treatments were continual for 2 months and all obese rats; except those on KD, were sustained under the obesogenic diet throughout the experimental period (the time-line of the study is indicated in **Figure 1**).

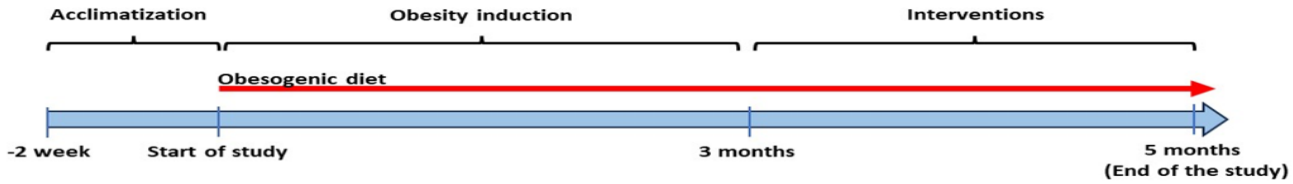

Figure (1): The time-line of the study

2.6. Collection of Samples

Overnight fasted rats were weighed at the end of the treatment period and blood samples were obtained from the tail tip to measure the fasting blood glucose (FBG) in the rats using an automatic glucose meter (Accu-Chek, Roche Diagnostics, Germany). Rats were subsequently decapitated after receiving intraperitoneal injections of xylazine (10 mg/kg) and ketamine (75 mg/kg) for anaesthesia **(Alsenousy et al., 2022).** To prepare the serum samples, blood was drawn from the retroorbital vein and placed in anticoagulant-free tubes. The blood was then centrifuged at 3000 ×g for 10 minutes. Insulin, lipid profile (triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C)), alanine aminotransferase (ALT) activity, and aspartate aminotransferase (AST) activity were measured using the serum samples. To extract total RNA for Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) study, liver tissues were collected, in order to assess the gene expression of PI3K, AKT, mTORC1, SREBP-1c.

2.7. Serum parameters measurements:

The homeostasis model assessment index for insulin resistance (HOMA-IR) was calculated using the following formula after the serum insulin concentration was measured using a rat-specific insulin ELISA kit (EMD Millipore, USA) in accordance with the manufacturer's instructions **(Caumo et al., 2006).**

$$\text{HOMA-IR}=\frac{\text{Fasting insulin}((\mu\text{IU})/\text{mL})\text{x}\text{Fasting glucose}(\text{mg/dL})}{22.5\times 18}$$

Using reagents obtained from BioMed Diagnostics INC (USA), the enzymatic colorimetric method was used to detect the serum levels of TG, TC, and HDL-C. Absorbance was evaluated at 546 nm. The following formula was used to determine serum LDL-C from the concentrations of TG, TC, and HDL-C. **(Tietz et al., 2006)**

LDL-C (mg/dL) = TC – (HDL-C) –TG/5

Using reagents obtained from BioMed Diagnostics INC (USA), serum ALT and AST activity were determined, and absorbance was measured at 340 nm **(Huang et al., 2006)**. Each procedure was carried out in compliance with the manufacturer's guidelines.

Serum level of BOHB was assessed using β-Hydroxybutyrate Colorimetric Assay Kit (Catalog No.: E-BC-K785-M, Elabscience, USA) according to the manufacturer's instructions.

2.8. Gene expression of PI3K, AKT, mTORC1, and SREBP-1c.

The RNeasy Mini Kit (Qiagen®, Germany) was used to isolate total RNA from liver in accordance with the manufacturer's instructions, and nanodrop was used to assess the extracted RNA's integrity and concentration. The miScript II RT Kit was used to do reverse transcription in accordance with the manufacturer's instructions. The tissue expression of PI3K, AKT, mTORC1and SREBP-1c were quantified in the cDNA relative to the reference gene 18s rRNA using Rotor Gene SYBR Green PCR Kit (Qiagen®, USA). The conditions for quantitative PCR amplification were set up as follows: a 10-minute initial denaturation at 95°C was followed by 45 PCR cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 70°C for 15 s. For normalisation, the housekeeping gene 18s rRNA used as a reference gene. **Table 2** lists the primers that were used to identify the rat genes. Using the 2-ΔΔCt method the relative change in mRNA expression in samples was determined **(Livak & Schmittgen, 2001)**.

Table (2): Primers sets for the gene expression of PI3K, AKT, mTORC1, SREBP-1c and reference gene 18s rRNA.

Gene name	Access No.	Primer sequence
PI3K	**NM_013005.2**	**F**: 5'-TGCTATGCCTGCTCTGTAGTGGT-3' **R**: 5'-GTGTGACATTGAGGGAGTCGTTG-3'
AKT	**NM_033230.3**	**F**: 5'-TCACCTCTGAGACCGACACC-3' **R**: ACTGGCTGAGTAGGAGAACTGG-3'
mTORC1	**NM_019906.2**	**F**: 5'- TTGGAGTGGCTGGGTGCTGA-3' **R**: 5'- AAGGGCTGAACTTGCTGGAA-3'
SREBP-1c	**NM_001276708.1**	**F**: 5'-GACGACGGAGCCATGGATT-3' **R**: 5'- GGGAAGTCACTGTCTTGGTTGTT-3'
NR_046237.2	**18s rRNA**	**F**: 5'- GTAACCCGTTGAACCCCATT -3' **R**: 5'- CAAGCTTATGACCCGCACTT -3'

2.9. Protein level of AKT, PI3K, mTORC1, and SREBP-1c.

Rat specific ELISA kits were used for measurement the hepatic protein contents of the active phosphorylated (p)-AKT (pSer473/474-Akt1/2) (cat. no. ADI-900-162, Enzo, USA), active phosphorylated (p)-mTOR (pSer2448-mTOR) (cat. no. ab168538, Abcam, USA), and PI3K and SREBP-1c proteins were assessed using rat-specific ELISA kits (Chongqing Biospes Co., Chongqing, China, Cat. No. BYEK2314 and BYEK3082) according to the instructions of the manufacturers. The Lowry method was utilized to determine the total protein concentration **(Classics Lowry et al., 1951).**

2.10. Statistical analysis

Version 18.0 of the SPSS software package (SPSS Chicago, IL, USA) was used to analyse the data. The data were presented as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA) for group comparisons. At $p < 0.05$, the p-value was considered significant. The Pearson correlation coefficient was utilised to assess the correlation coefficients (r) across different parameters under investigation. A significance limit of $P<0.05$ was applied to all comparisons **(Hagen, 2002)**.

3. Results

3.1.3.1. Body weight change

All obese groups are significantly heavier than the control rats at the start of treatments with no significant differences between the obese groups. The final body weights show similar pattern of change but the data show high degree of dispersion as indicated by the high standard deviations (SD) (Table 3). The untreated obese rats showed significantly higher weight gain as compared to the control rats. The treatment of the obese rats with orlistat and/or BOHB noticed lower body weight gain as compared to the untreated rats but the differences not significant. The obese rats maintained under KD showed significantly lower body weight gain as compared to the other obese groups and lower than the control rats but not significantly (Table 3). Three rats of the KD group showed weight loss (not gain) during the study period (data not shown).

Table (3): Changes of the initial weight, final weight and weight gain in the studied groups.

	Groups	Initial Weight (g)	Final Weight (g)	Weight gain (g)
	Control	229.6^{b} ±9.94	250.0^{b} ±11.41	20.38^{bc} ±5.76
Obese	**Untreated**	316.1^{a} ±55.87	396.5^{a} ±73.69	80.38^{a} ±32.41
Obese	**Orlistat**	323.4^{a} ±21.22	379.1^{a} ±29.59	55.75^{ab} ±27.64
Obese	**BOHB**	321.6^{a} ±49.78	388.8^{a} ±61.40	67.13^{a} ±49.96
Obese	**Orlistat + BOHB**	317.9^{a} ±51.90	363.1^{a} ±76.24	45.25^{abc} ±26.92
Obese	**Keto-diet**	321.4^{a} ±25.66	328.9^{a} ±23.34	7.50^{c} ±18.57

Data was expressed as Mean ± SD, and n=8

*Means in the **same column** with **common superscript letters** are not significantly differ and means with **different superscript letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.*

3.2. Glucose homeostasis parameters:

When compared with the normal rats, the level of FBG in obese (untreated) rats was significantly increased. All the obese (treated) rats had complete normalization of the FBG which significantly lower as compared to the obese (untreated) rats and there was non-significant difference between the obese (treated) rats **(Table 4).**

The insulin level was significantly higher in obese (untreated) rats in comparison with the normal rats. Orlistst- treated rats did not have significantly effect on the insulin in comparison with obese (untreated) rats. Compared to the obese (untreated) rats, the insulin levels of the obese rats treated with BOHB (alone or combined with orlistat) are significantly reduced and there was non-significant difference compared with the normal rats **(Table 4).**

The homeostasis model assessment index for insulin resistance (HOMA-IR) was markedly higher in the untreated obese rats as compared to the normal group. All the obese (treated) groups showed significantly decline HOMA-IR in comparison with the obese (untreated) rats and there was non-significant difference between the treated rats **(Table 4).**

Table (4): Changes of glucose homeostasis parameters in the studied groups

	Groups	FBG (mg/dl)	Insulin (µIU/ml)	HOMA–IR
	Control	104.5^{b} ±10.64	3.94^{c} ±1.42	1.04^{b} ±0.43
Obese	**Untreated**	167.9^{a} ±19.87	15.49^{a} ±7.14	6.45^{a} ±2.90
Obese	**Orlistat**	122.9^{b} ±10.91	9.94^{ab} ±3.90	3.0^{b} ±1.20
Obese	**BOHB**	123.0^{b} ±25.33	8.89^{bc} ±3.71	2.71^{b} ±1.41
Obese	**Orlistat +BOHB**	127.8^{b} ±27.51	6.77^{bc} ±2.40	2.18^{b} ±1.0
Obese	**Keto- diet**	134.6^{b} ±20.71	7.24^{bc} ±2.85	2.46^{b} ±1.09

Data was expressed as Mean ± SD, and n=8

*Means in the **same column** with **common superscript letters** are not significantly differ and means with **different superscript letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.*

MVC (-): without macrovascular complications, MVC (+): with macrovascular complications

3.3. Lipid profile parameters:

The obese (untreated) rats have significantly elevation in triglycerides (TG) level as compared to the normal rats. Only the orlistat-treated rats had significantly reduced TG level as compared to the obese (untreated) rats. The obese rats feed KD showed no changes in the TG compared to the untreated group while the treated rats with BOHB combined with orlistat showed mild but not significant decline **(Table 5).**

Regarding the total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C), the obese (untreated) rats had a significantly elevated levels compared with the normal group. All the treated rats had significantly lower TC and LDL-C levels as compared to the obese (untreated) rats. The best lowering effects noticed in the rats treated with orlistat and/or BOHB, which showed lower TC levels than the normal group while the obese rats feed KD had normalized levels **(Table 5).**

Obese (untreated) rats had significantly lower high-density lipoprotein-cholesterol (HDL-C) levels in comparison with the normal rats. All treated groups had no significant changes in the HDL-C as compared to the untreated group except rats treated with combination of orlistat and BOHB which showed further significant decline in HDL-C as compared to the obese (untreated) group **(Table 5).**

3.4. Serum activities of alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST) aminotransferase activity (AST)

All obese rats have significantly higher ALT activity compared with normal rats with non- significant differences between the obese rats. Regarding AST activity, untreated obese rats have significantly higher AST activity as compared to normal rats. Only the obese rats treated with BOHB (alone or combined with orlistat) have significantly lower AST activity in comparison with the obese (untreated) rats **(Table 5).**

Table (5): Changes of lipid profile parameters and liver function tests parameters in the studied groups

Groups		Triglycerides (mg/dl)	Total cholesterol (mg/dl)	HDL–c (mg/dl)	LDL–c (mg/dl)	ALT (U/L)	AST (U/L)
	Control	37.63^{c} ±3.11	25.0^{a} ±5.18	88.48^{b} ±13.02	36.75^{b} ±4.40	122.0^{b} ±12.56	121.0^{b} ±9.23
Obese	**Untreated**	97.13^{a} ±11.26	19.75^{b} ±4.10	129.0^{a} ±9.90	61.25^{a} ±16.10	173.1^{a} ±14.16	168.1^{a} ±8.97
	Orlistat	69.88^{b} ±12.71	16.29^{bc} ±1.58	33.61^{c} ±11.46	56.63^{a} ±5.83	179.3^{a} ±15.85	63.88^{c} ±12.82
	BOHB	88.25^{ab} ±10.14	18.88^{bc} ±1.27	43.85^{c} ±4.95	65.88^{a} ±14.60	92.25^{c} ±14.83	80.38^{c} ±7.80
	Orlistat+ BOHB	77.75^{ab} ±18.74	14.83^{c} ±1.85	44.87^{c} ±13.90	53.75^{a} ±4.33	91.75^{c} ±14.91	75.25^{c} ±14.05
	Keto-diet	92.50^{a} ±23.69	17.78^{bc} ±2.24	72.97^{b} ±11.36	64.25^{a} ±9.53	168.0^{a} ±19.65	109.3^{b} ±12.91

Data was expressed using Mean ± SD. n=8, Means in the **same column** with **Common letters** are not significantly differ (i.e. Means with **Different letters** are significantly differ

3.5. Serum level of BOHB

The serum level of BOHB showed no significant changes in obese (untreated) rats or orlistat-treated rats while its level significantly increased in all obese groups treated with BOHB (alone or combined with orlistat) or treated with KD (**Figure 2**).

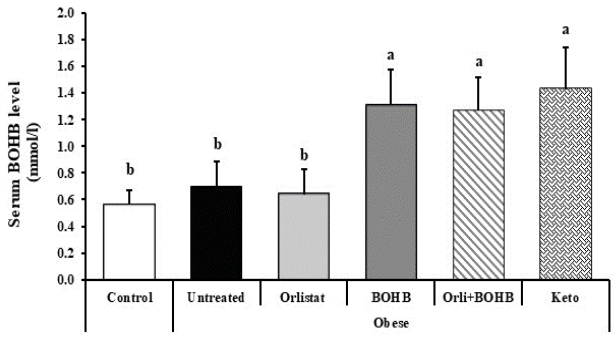

Figure (2): Serum BHOB levels in the studied groups. Data presented as Mean±SD and n=8. Means with common letters are not significantly differ and means with different letters are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.

3.6. Hepatic expression of PI3K at mRNA and protein levels

The hepatic tissue of the untreated obese rats showed significant downregulated PI3K expression by about 40% in comparison with the normal group. All the treated groups have significant upregulation in the hepatic expression of PI3K as compared to the obese (untreated) group with the highest expression noticed in the KD rats followed by the rats treated with BOHB alone or combined with orlistat **(Figure 3A).**

At protein level, the hepatic PI3K showed opposite pattern of change compared to its mRNA pattern. The untreated obese rats had significant elevation in the hepatic protein level of PI3K as compared to the normal rats. Only the obese group feed with KD had significant correction and completely normalized the hepatic protein level of PI3K (**Figure 3B**).

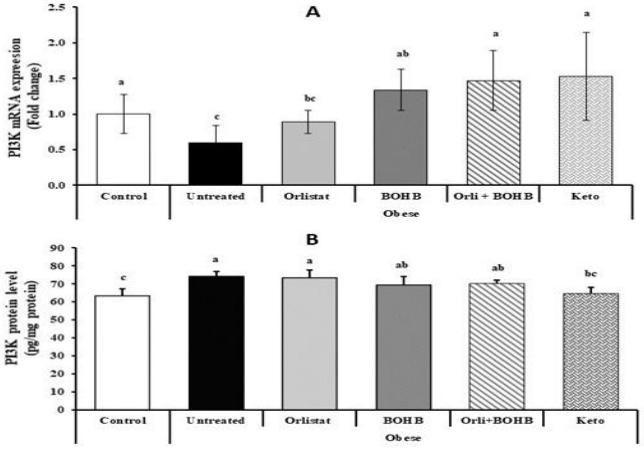

Figure (3): Hepatic expression of PI3K at (A) mRNA and (B) protein levels in the studied groups. Data presented as Mean±SD and n=8. Means with **common letters** are not significantly differ and means with **different letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.

3.7 Hepatic expression of protein kinase B (AKT) at mRNA and protein levels

The obese (untreated) group had a significant downregulation in the hepatic expression of AKT expression by about 64% in comparison with the normal group. Only the obese rats feed KD showed significant upregulation in the hepatic expression of AKT compared with the untreated rats, while other treatments fail to show any significant change in the expression of AKT **(Figure 4A).**

At protein level, active phosphorylated AKT (p-AKT) showed opposite pattern of change compared to its mRNA pattern. The untreated obese rats had a significant higher level of the hepatic protein of p-AKT as compared to the normal group. The treatment of obese rats with BOHB (alone or combined with orlistat) or KD significantly corrected the hepatic p-AKT level and completely normalized in the rats feed with KD while orlistat-treated rats alone showed no significant change **(Figure 4B).**

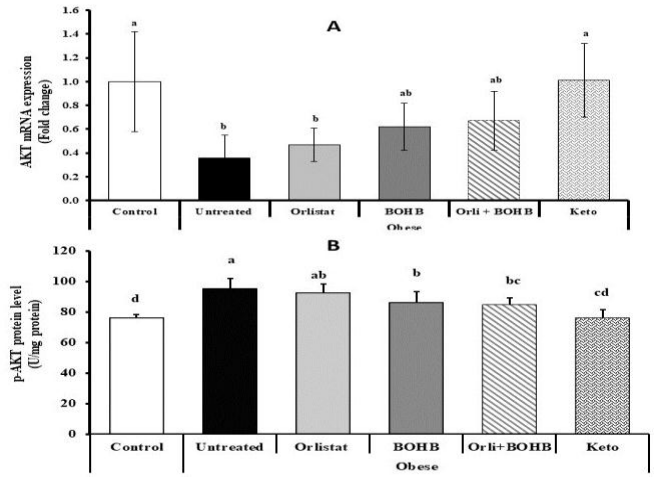

Figure (4): Hepatic expression of AKT at (A) mRNA and (B) active phosphorylated protein levels in the studied groups. Data presented as Mean±SD and n=8. Means with **common letters** are not significantly differ and means with **different letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.

3.8. Hepatic expression of mammalian target of rapamycin complex 1 (mTORC1) at mRNA and protein levels

The obese (untreated) group had a significant upregulated expression of the hepatic mTORC1 at mRNA level in comparison with the normal group. While the treatment with orlistat alone didn't significantly affect the mRNA level compared with the untreated group, the treatment with BOHB (alone or combined with orlistat) or with KD noticed significant downregulation in the hepatic expression of mTORC1 as compared to the obese (untreated) rats and completely normalized its expression **(Figure 5A).**

The obese (untreated) group showed significant elevation of the hepatic active phosphorylated mTOR at ser2448 (p-mTOR) protein level as compared to the normal group. All the treated groups significantly reduced the hepatic p-mTOR in comparison with the obese (untreated) rats. The best results obtained in obese rats feed with KD which had no significant difference with the control rats **(Figure 5B).**

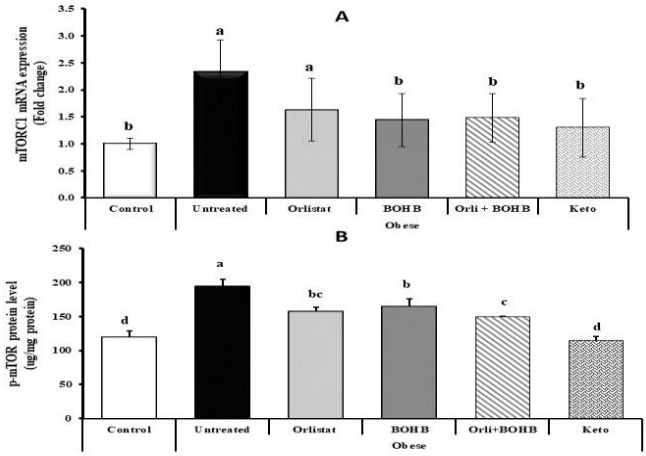

Figure (5): Hepatic expression of mTORC1 at (A) mRNA and (B) protein levels in the studied groups. Data presented as Mean±SD and n=8. Means with **common letters** are not significantly differ and means with **different letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.

3.9. Hepatic expression of sterol regulatory element binding protein (SREBP-1c) at mRNA and protein levels

The obese (untreated) rats and orlistat- treated rats have significantly upregulated expression of SREBP-1c in liver in comparison with control rats. The KD rats had significantly downregulated expression of SREBP-1c as compared to the obese (untreated) rats and had completely normal expression. The obese rats treated with BOHB (alone or combined with

orlistat) showed mild downregulation in the expression of SREBP-1c in liver as compared to the obese (untreated) rats **(Figure 6A).**

The obese (untreated) group had significantly higher protein level of SREBP-1c in liver as compared to normal group. All treated rats have significantly lower hepatic SREBP-1c protein compared with the obese (untreated) rats. The obese rats feed with KD had non-significant difference with the control rats **(Figure 6B).**

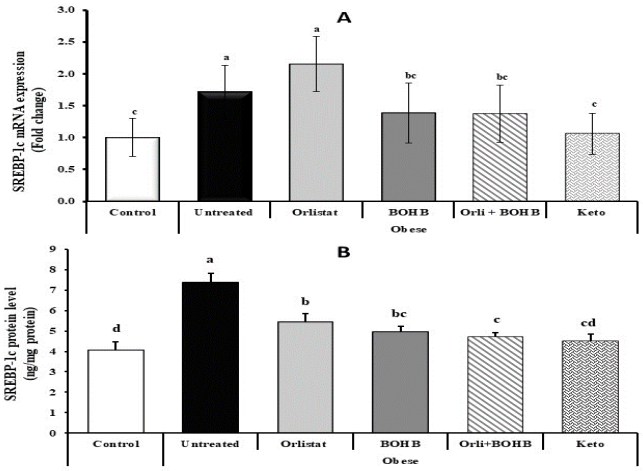

Figure (6): Hepatic expression of SREBP-1c at (A) mRNA and (B) protein levels in the studied groups. Data presented as Mean±SD and n=8. Means with **common letters** are not significantly differ and means with **different letters** are significantly differ by ANOVA followed by Tukey post-hoc test, $p<0.05$.

4. Discussion

The KD showed potential anti-obesity effects associated with amelioration of carbohydrates and lipid metabolism in addition to improvement of hepatic AKT/PI3K/mTOR pathway in rats with HFD-induced obesity. The HFD-obese rats in this study showed the main characteristics of obesity in humans including, weight gain, insulin resistance and dyslipidemia. These derangements are accompanying with marked hepatic molecular changes such as suppressed expression of PI3K/AKT at mRNA level while enhanced at protein level, and enhanced expression of the lipogenic SREBP-1c and mTORC1. These epigenetic effects are suggested to induce marked metabolic shift in the hepatic tissues into induced proteins and lipids synthesis. The conflict results of PI3K and AKT at mRNA and protein levels could be elucidated by the presence of another post-transcriptional and/or post-translational mechanisms which may suppress mRNA expression as a feedback inhibition mechanism to counteract the enhanced AKT/PI3K pathway in obesity and NAFLD that previously reported **(Matsuda et al., 2013; Savova et al., 2023; Sun et al., 2023)**. This suggestion needs further investigations.

Insulin resistance, dyslipidemia, inflammation, adipose tissue growth, hyperinsulinemia, and/or glucose intolerance are all part of the vicious cycle which transitions from a metabolically healthy state to an obese and prediabetic state **(Kim et al., 2001)**. These metabolic abnormalities in the obese rats result in a significant raise in the activities of transaminases; AST and ALT in serum which may indicate hepatic inflammation and hepatocytes damage.

Today, the application of ketogenic diet (KD) for the control of obesity is widely used all over the world. The KD is a diet that is high in fat, low in carbohydrates, and sufficient in protein; as a result, it fully contradicts the conventional dietary guidelines on the composition of the macronutrient pyramid **(Bueno et al., 2013)**. This KD push the metabolism towards ketogenesis (ketone bodies synthesis and utilization). This factor has a significant impact on the individuals' quality of life and compliance with KD treatment. The process of ketogenesis, which largely takes place in the liver, uses the mitochondrial acetyl-CoA pool to synthesise the two primary ketone bodies, acetoacetate and BOHB **(d'Avignon et al., 2018).** In the current study feeding the obese rats with KD or treatment with BOHB (alone or in combination with orlistat cause a significant elevation in the serum level of BOHB in a condition called nutritional or physiological ketosis which is safe because ketone bodies are produced in low levels that cannot change blood pH, which is very different from the condition of pathologic ketoacidosis, which is a life threatening due to production of high amounts of ketone body resulting in changing the blood pH to become acidic **(Masood & Uppaluri, 2019).**

The anti-obesity effect of KD compared with the conventional used drug; orlistat and the possible anti-obesity effects of BOHB alone or combined with orlistat were examined in this study. The results showed that feeding the obese rats with KD had a significant reduction in the final body weights and weight gains of the obese rats. While the orlistat and BOHB treatments dose not significantly ameliorate the final body weights and weight gains of obese rats. Only the obese rats which received combination of orlistat and BOHB showed weight gain with no significant difference from the control group.

In addition, the KD significantly ameliorated and completely normalized the hyperglycemia, insulin level, and insulin resistance in the obese rats. Also, BOHB alone or combined with orlistat significantly ameliorated the glucose homeostasis parameters like or even better than the treatment with orlistat alone. The hypoglycemic effect of KD may result from decreased glucagon as reported **Granados-Rojas et al. (2020)**, which might result in reduced gluconeogenesis in the liver and shield KD-fed animals from hyperglycemia **(Gupta et al., 2017)**. In morbidly obese adults with a body mass index (BMI) of ≥45 kg/m, short-term KD combined with a nearly carbohydrate-free diet may lower blood pressure, insulin resistance, waist circumference, and body weight **(Castaldo et al., 2016)**.

Orlistat significantly improved the lipid profile, which is partly mediated by its lipotropic action by significantly decreasing the triglycerides as compared to the untreated

obese rats, and significantly declining the total, LDL-, and HDL-cholesterol compared with the untreated obese rats and control rats. The obese rats treated with BOHB alone or combined with orlistat showed no significant changes in the triglycerides level compared with the untreated rats and showed significant decline in the levels of total, LDL- and HDL-cholesterol. Regarding the obese rats feed KD, the triglycerides and HDL-cholesterol levels had no significant difference compared with the untreated rats while the total and LDL-cholesterol levels showed near normal values.

Considering the effect KD, and BOH on total cholesterol and LDL-cholesterol, they might be recommended as a hypolipidemic agent, which would be very beneficial for obesity. However, they have no significant effects on the triglycerides and significantly exaggerated the decline in the HDL-cholesterol that may considered a risk for cardiovascular diseases. In obese hypercholesterolemic patients with a BMI > 35 kg/m2, a long-term study found that the KD significantly lowered BMI, total cholesterol, and fasting glucose. It also enhanced weight reduction, which in turn reduced the risk factors for obesity-related disorders **(Dashti et al., 2004).** Another controlled study enrolling 20 patients receiving KD for 8 weeks, showed significant improvement in BMI, insulinemia, TGs, LDL-C, and liver transaminase. Similarly, a meta-analysis study demonstrated that compared to low-fat diets, KD more successfully improved the metabolic parameters linked to glycemic, weight, and lipid control in obese participants, especially those who with preexisting diabetes. **(Choi et al., 2020)**. Nonetheless, the primary major issue with KD in the treatment of obesity is plasma lipids. The KD has a beneficial impact on both total and LDL cholesterol, according to multiple lines of evidence. The majority of research shows that eating less carbohydrates can raise HDL-C levels while lowering TG and total cholesterol **(Bueno et al., 2013; Paoli et al., 2013)**.

The precise molecular mechanism (s) included in the anti-obesity action of KD on insulin sensitivity is incompletely understood. Several mechanisms have been proposed including: (1) reduced appetite by elevating the levels of "satiety" hormones (glucagon-like peptide-1, cholecystokinin, and ghrelin) **(Veldhorst et al., 2008; Veldhorst et al., 2009)** and by ketone bodies' direct reduction of appetite, as BOHB , which interfere with the central energy and satiety signaling **(Johnstone et al., 2008; Laeger et al., 2010)** (2) inhibited lipogenesis because of improvement of insulin resistance **(Yang et al., 2021)** and enhanced lipolysis by upregulated expression of lipolytic enzymes, (3) Greater metabolic efficiency when consuming fats **(Paoli et al., 2012; Tagliabue et al., 2012)**, and (4) Thermic effect of proteins (dietary proteins-induced thermogenesis), which has the highest energy cost among the three macronutrients, and increased gluconeogenesis, an energy-intensive process that costs approximately 400–600 Kcal/day **(Fine & Feinman, 2004; Feinman & Fine, 2007).**

In the current study, the serum transaminases; ALT and AST showed different pattern of changes during the treatment of obesity in the rats. The high ALT activities in the obese rats does not significantly affected by the different treatments used in the present study while the AST activity showed significant decline in the obese rats received BOHB alone or combined with orlistat while the KD dose significantly affect the AST activity which may imply that KD does not ameliorate the hepatic impact of obesity. It was reported that weight loss, amelioration in insulin resistance, and reduction of hyperlipidemia may help to improve liver diseases progression (Dyson et al., 2014). So, the KD may ameliorate the obesity-induced liver damage by promoting weight loss and insulin sensitivity. It was noted, therefore, that prolonged KD maintenance induced the development of both glucose intolerance and liver fibrosis **(Schugar & Crawford, 2012).**

At the molecular level, the KD feeding of obese rats significantly corrected the disturbed expression of PI3K, AKT, mTORC1 and SREBP-1c at both mRNA and protein levels. Surprisingly, the treatment of obese rats with BOHB alone or in combination with orlistat demonstrated significant correction of the disturbed mRNA and protein levels of the studied genes. On the other hand, the orlistat treatment does not affect the expression of these genes as compared to the untreated obese rats. These data are in harmony with many reports that confirm the suppression of mTORC1 by KD **(McDaniel et al., 2011).** Also, KD has positive effects on epilepsy and Alzheimer's disease through suppressing the expression of mTORC1. It has been discovered that KD is useful in treating and preventing type 2 diabetes in people. Ketoneuria during spontaneous (overnight) fasting was linked to a lower risk of diabetes in a study of healthy participants, suggesting that spontaneous ketosis may avert the onset of diabetes **(Kim et al., 2019)**. The aged mice have suppressed ketogenesis and rapamycin (the inhibitor of mTORC1) increases ketones production **(Sengupta et al., 2010)**.

The KD is reported to elevate adenosine monophosphate-activated protein kinase (AMPK) activity which may inhibit the mTORC1 pathway and may affect the expression of PI3K/AKT **(McDaniel et al., 2011)**. Recently, **(Zhou et al., 2022)** It was discovered that by downregulating hepatic expression and activating SREBP-1c, KD and BOHB may ameliorate lipid dysregulation in type 2 diabetic mice. Giving the prominent role of SREBP-1c in lipogenesis and obesity make it one of the main targets of obesity treatments. It is well known that SREBP-1C's stability and activity were enhanced by acetylation **(Ponugoti et al., 2010)**. According to Zhou et al., It was proposed that by preventing the acetylation of SREBP1c, BOHB and KD significantly decreased its activity **(Zhou et al., 2022).**

The effect of KD on the gene expression may be mediated through the direct effects of ketone bodies, especially BOHB **(Youm et al., 2015).** Through several ways, BOHB may connect the metabolic environment to epigenetic control and cellular function. Its roles as a passive energy transporter are complemented by a variety of direct and indirect signalling activities that impact metabolic rate, lipid metabolism, gene expression, and neuronal function. These effects include protein posttranslational modification (lysine β-hydroxybutyrylation), competitive inhibition of certain enzymes, and binding to cell-surface receptors—all direct signalling actions of BOHB. **(Newman & Verdin, 2017).** Class I histone deacetylases (HDACs), a family of proteins

that deacetylate lysine residues on histone proteins to restrict gene expression, may be inhibited by BOHB. HDAC inhibitors have been used to treat metabolic disorders by preventing tumour growth **(Chang & Min, 2002)** and neurological disorders **(Yoo & Ko, 2011)** moreover HDAC inhibitors can extend the lifespan of experimental animals **(Kang et al., 2002).** Interestingly, p53 hyperacetylation was found to inhibit mTORC1 in response to fasting **(Schupp et al., 2013)** and feeding KD increased p53 acetylation and decreased mTORC1 downstream signaling. Furthermore, KD has been shown in animal studies to impact multiple pathways involved in metabolic syndrome and cancer. These pathways include raising the expression of peroxisome proliferator-activated receptor-γ coactivator-1α (a master mitochondrial metabolic regulator that can increase mitochondrial biogenesis) and decreasing the serum ratio of insulin-like growth factor (IGF)/IGF-binding protein **(Srivastava et al., 2012).** Additionally to inhibiting HDAC, BOHB has the ability to alter proteins at the posttranslational level by lysine β-hydroxybutyrylation. Histone β-hydroxybutyrylation has been shown to rise in human cells in direct proportion to exogenous BOHB administration, and in mouse liver tissue in response to rises in plasma BOHB levels following either normal mouse fasting or diabetic rat ketoacidosis **(Xie et al., 2016)**.

The BOHB appears to have mechanistic role in the action of KD because it can improve glycemic control, insulin levels, insulin resistance, and lipid profile in obese rats before significant weight loss occurs, it can have a number of positive effects on obesity. This suggests that the BOHB may improve metabolic markers independently of weight loss and may even play a direct role in the anti-obesity effects of KD. The brain, adipose tissues, and heart are among the metabolically important organs that are impacted by BOHB in a variety of ways. These effects may be crucial for the anti-obesity or adverse effects of KD or BOHB. While numerous studies show that KD, at least initially, leads to effective weight loss, the degree of weight gain over the long term determines the effectiveness of any nutritional strategy **(Janda et al., 2013).**

5. Conclusion

The KD and BOHB have potential anti-obesity effects through the amelioration of glucose and lipid homeostasis, insulin sensitivity and hepatic PI3K /AKT/mTOR pathway.

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The authors declare no conflict of interest.

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