Research article

Urinary metabolites of type 2 diabetes rats fed with palm oil-enriched high fat diet

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ABSTRACT

High fat diet (HFD) is one of the risk factors of obesity and diabetes. Recommended diet regimen for diabetes is difficult to abide by especially for HFD as it adds flavour to the taste buds. In this study, palm oil-enriched HFD and normal diet were fed to nicotinamide-induced type 2 diabetes rats, respectively for six weeks. Additionally, metformin, a common drug used to treat diabetes was given to rats under treatment groups. We evaluated the change of urinary metabolites of diabetes rats fed with palm oil-enriched HFD, and also after metformin treatment. Rats were divided into six-groups with different feeding diets, disease condition and with or without metformin treatment. Rats' urine were collected at the end of six weeks feeding program and subjected to 1H-NMR and multivariate data analysis to evaluate their metabolite profiles. At the early phase of diabetes, metabolites changes in diabetic rats were associated with the disease itself. Our data showed that continuous consumption of HFD altered various metabolic pathways of diabetic rats and caused detrimental effects to the rats. On the other hand, metformin treatment combined with normal diet lessened the physiological impacts caused by diabetes condition.

1. Introduction

Diabetes is one of the most common non-communicable diseases affecting 9.3% of world population, it is characterised by high blood glucose levels [1]. Type 2 diabetes mellitus (T2DM) is the most common type of diabetes. Obesity is strongly associated with T2DM as it is one of the leading causes of insulin resistance, a typical characteristic of T2DM [2]. The most common cause of obesity is due to high fat diet (HFD) consumption. HFD is defined as the dietary fat intake >30% of the total diet [3] and it has been linked to metabolic diseases, including obesity, diabetes, and cardiovascular diseases.

Palm oil is the most consumed fat in Asia, Africa, and Europe [4, 5]. Palm oil is a very popular household cooking oil and ingredient in food industry due to its texture, fragrance, and neutral taste compared to other vegetable oils. Besides, palm oil has a high smoke point at 230 °C, making it suitable for most cooking methods [5]. Palm oil has a balanced ratio of unsaturated and saturated fatty acids. Palmitic acid is the major saturated fatty acid in palm oil (45%) and followed by stearic acid (5%). Additionally, palm oil also contains 40% oleic acid (monounsaturated fatty

acid) and 10% linoleic acid (polyunsaturated fatty acid) [4]. Palmitic acid (16:0), a long chain saturated fatty acid is the most abundant saturated fatty acid present naturally in vegetable oil, animal fat, and human milk fat [6]. Among the fatty acids, long-chain saturated fatty acid, such as palmitic acid, was said to be lipotoxic compared to medium-chain (C6–C12) saturated fatty acids or unsaturated fatty acids [7, 9]. Long-chain saturated fatty acid was reported to promote insulin resistance, inflammation, and fat storage [7, 8, 9]. Nevertheless, a recent study has shown that long chain saturated fatty acids were well tolerated by human β-cells and therefore may not cause insulin resistance [9].

Besides dietary fat, lifestyle modifications such as healthy eating and physical activity, are essential for diabetes management [10]. When lifestyle interventions fail or it becomes hard to manage the blood glucose levels, medical treatment becomes an important option. Metformin (dimethylbiguanide) is one of the most common drugs, and the first-line treatment for T2DM [11]. Metformin is an antihyperglycemic agent that decreases hepatic glucose production, increases peripheral glucose uptake, and halts gastrointestinal glucose absorption [12].

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In this study, NMR-based metabolomics approach was applied to evaluate the urinary metabolites. This technique consists of a combination of 1H-NMR spectroscopic profiling and multivariate statistical analysis. It is a powerful tool for identifying changes in metabolites profiles of biological samples. It is a common approach used in diet and drug intervention for diseases [13]. Urine has high medical diagnostic and biomarker values as it does not involve body homeostasis; it can be collected easily and non-invasively [14, 15]. Biomarkers related to diet, or food intervention have been discovered via urinary metabolomics-based analysis. For instance, proline betaine and hydroxyproline betaine are biomarkers of citrus juice consumption, while creatinine, carnitine, acetylcarnitine and trimethylamine-N-oxide are the biomarkers of red meat consumption. On the other hand, formate, citrulline, taurine and isocitrate are used as markers for sugar sweetened beverages [16, 17, 18, 19]. Furthermore, urine metabolomics is useful to detect endogenous metabolic variation and kidney function efficiency. This is an added value to our study because the kidneys are commonly affected by diabetes.

Although studies on the metabolites profile of diabetic rats [20, 21, 22, 23, 24] had been reported, where the pathogenesis and progression of the disease were evaluated by elucidation of urinary metabolites profile. Nevertheless, study on urinary metabolites changes due to palm oil-enriched HFD on diabetic rats is new to our knowledge. We think this information is important because diabetes cases are on the rise as palm oil is heavily used in cooking preparations, especially frying, which adds taste and texture to the palate [25]. Not to mention, these food can hardly be resisted by diabetes patients. Therefore, we aimed to evaluate the impact of palm oil-enriched HFD upon T2DM rat models' urinary metabolite profiles using 1H-NMR and multivariates data analysis, which was hoped to provide a deeper understanding on the impact of palm oil-enriched HFD to the existing metabolic pathways of the disease.

2. Materials and methods

2.1. Animal study

Male Sprague Dawley rats weighing 300 ± 50 g were obtained from Animal Research Center and Service, Universiti Sains Malaysia. The rats were housed individually in plastic cages with 12 h of light/dark cycles and were acclimatised for a week before the study started. All experiment procedures were approved by the Animal Ethics Committee Universiti Sains Malaysia (USM/Animal Ethics Approval/2016/(717)).

2.2. Induction of diabetes mellitus

The rats were induced to diabetes by nicotinamide (Sigma, USA) and streptozotocin (Sigma, USA) according to Akbarzadeh et al (22). Nicotinamide dissolved in saline (110 mg/kg of rat body weight) was administered to the rats through intraperitoneal injection. After 15 min, streptozotocin (65 mg/kg of rat body weight), which was dissolved freshly in 0.1 M sodium citrate dihydrate (Sigma, USA), pH 4.5 was injected intraperitoneally to the rats. Control rats were given only the vehicle solution (saline and citrate buffer). The rats were monitored for four weeks before separated into different groups. Rats with fasting blood glucose levels >7 mmol/L were classified as diabetic rats. The experimental groups are shown in Table 1.

2.3. Diet/feed

The normal diet (ND) and HFD were self-prepared by mixing distilled water with the commercial feed powder (contained 12 % of fat content) (Altromin, Germany) at 1:1 ratio and baked in an oven overnight at 40 °C. HFD was prepared by adding 22.4 % (v/w) palm olein cooking oil (Buruh Nam Loong, Malaysia), making a total of 39 % fat content. The final feed products were sent for further analysis and the final composition of the feeds was obtained. Metformin HCl Tablet 500 mg

Table 1. The experimental group for the study.

Group	Non-induced/Induced rat	Diet	Metformin	n
NN	Control	ND	No	6
NH	Control	HFD	No	6
DN	Diabetic	ND	No	6
DNM	Diabetic	ND	Yes	6
DH	Diabetic	HFD	No	6
DHM	Diabetic	HFD	Yes	6

NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat fed with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin).

(Dynapharm, Malaysia) was crushed and mixed with 1 % carboxymethylcellulose (R&M Chemicals). The oral gavage was given twice daily. Vehicle (1 % carboxymethylcellulose) was given to the rats that were not given metformin. The feeding programme was 6 weeks. Water (ad libitum) was provided throughout the study.

2.4. Fasting blood glucose

Fasting blood glucose was measured for every rats in the groups (n = 6). The measurement was first taken a week before nicotinamide/streptozotocin induction (P1) and then weekly from the starting point of feeding program until the sixth week of feeding program (W0 to W6) by using Glucometer (Accu-chek Performa, Roche). A drop of blood was taken from the rat tail. The rats were fasted for 12 h before measurement was taken.

2.5. Urine collection

At the end of the feeding period (6 weeks), urine was collected by placing the rat in a metabolic cage for 24 h. A volume of 500 μl of 1 % sodium azide (Sigma, USA) was added to the urine collection bottles. The urine was stored at -80 °C until further analysis.

2.6. Sample preparation for 1H-NMR analysis

The urine samples were thawed. The thawed urine was centrifuged at 8000 rpm at 4 °C for 5 min. A volume of 400 μl of the supernatant was pipetted to a new Eppendorf tube and mixed with 200 μl of phosphate buffer. The phosphate buffer contained 28.85 g disodium hydrogen phosphate, Na_2HPO_4 (R&M Chemicals, UK), 5.25 g sodium dihydrogen phosphate, NaH_2PO_4 (R&M Chemicals, UK), 1 mM (0.172 g) sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4, TSP (ARMAR AG, Switzerland) and 200 ml of deuterium oxide, D_2O (ARMAR AG, Switzerland). The volume was topped up to 1 L by distilled water. Urine and phosphate buffer mixture were vortexed for a few seconds. Finally, 550 μl of the mixture was transferred to a 5 mm NMR tube and capped.

2.7. 1H-NMR analysis

1H-NMR spectra of urine were collected without magic-angle spinning at $300K \pm 0.1$ on a Bruker Advance III 500 MHz (Bruker, Fallanden, Switzerland) spectrometer with a room temperature (25 °C) observe detection probe equipped with z-gradients and an automatic sample changer. The temperature was calibrated using 99.8 % deuterated methanol. Each sample was held for 5 min (300 s) in the magnet for thermal equilibration before measurement. The probe was tuned and matched automatically and the magnetic field was locked to the solvent (urine + D_2O), optimised for urine and shimmed using an automatic routine. Automatic 1H pulsecal calibration (pulsecal) was performed on each sample to avoid the effect of sample variation. Each sample was subjected to 32 scans with 4 dummy scans, 0.16 Hz/point and relaxation

delay (D1) of 4 s. Water suppression was achieved to suppress water signal at 4.74–4.90 ppm through weak radio frequency radiation during recycle delay. Water residual was further cleaned up by pulse field gradient. The spectra were recorded and processed using TopSpin 3.2 (Bruker Fallanden, Switzerland). The spectra were processed according to pre-set automated routine where the spectra were phased, baseline corrected and calibrated to TSP at 0.0 ppm. Automatic zero-order phase correction was applied while first-order phase correction was optimised and adjusted according to Bruker standard during setup. All samples followed the same acquisition and processing parameters. The spectrum's quality was attained by checking the line width at half height of TSP peak. It was considered satisfactory if the value was <2 Hz.

2.8. Multivariate data analysis and metabolites identification

NMR data spectrum was reduced and segmented into 250 bins of 0.04 ppm width from the region of 0.02–10 ppm using AMIX software (Bruker Analytische Messtechnik, Rheinstetten, Germany). NMR data were normalised to total peak intensity and scaled to TSP (reference region). The Amix file was exported to Microsoft Excel for SIMCA analysis to generate OPLS-DA. Water (4.74–4.90 ppm) and urea regions (5.70–5.90 ppm) were excluded from the analysis to avoid effect of inter-sample variation due to water suppression. Glucose regions (3.22–3.98 ppm, 4.62–4.66 ppm, and 5.18–5.30 ppm) of the diabetic rats was excluded. For the comparisons that involved metformin treated groups, metformin region (3.038–3.055 ppm) was excluded in OPLS-DA analysis. The data were scaled using auto Pareto scaling in SIMCA-P 13 (Umetrics, Umea, Sweden) and OPLS-DA analysis, from which a VIP plot was generated to identify the important regions that contributed to the difference between the groups. Only the NMR peaks in regions with VIP score >1 were integrated, and subsequently a second OPLS-DA analysis was carried out using the integrated data. Identification of the metabolites with VIP score >1 was carried out by matching of their respective chemical shift, multiplicity (singlet, doublet or quartet) and relative intensity values with the values of known metabolites in the databases from Biological Magnetic Resonance Bank (BMRB), Human Metabolome Database (HMDB) and Chenomx profiler. A match indicates the identity of the metabolite.

3. Results

3.1. The effect of HFD and metformin on fasting blood glucose

In this study, all the rats were with normal fasting blood glucose (FBG) < 5.5 mmol/L before the induction of diabetes by nicotinamide

and streptozotocin. Figure 1 shows the chart for the blood glucose of rats (n = 6) in each groups (mean ± SD). The parameter to define diabetes condition was FBG >7 mmol/L [26, 27]. A drastic increase in FBG (p > 0.05) within the first week of HFD feeding program was observed in diabetic rats, while for the ND fed diabetes rats, a gradual increase in FBG was observed. The FBG for diabetic rats reached a plateau at 19.4–22.9 mmol/L throughout the remaining feeding period (up to week-6). Metformin treatment reduced FBG of diabetic rats to a level ranged between (11.0–16.4 mmol/L). HFD did not alter the FBG of control rats throughout the feeding program.

3.2. The effect of HFD on urinary metabolites of control and diabetic rats

Table 2 shows the comparison between groups of animals that was carried out in this study. The effect of HFD on the rats was carried out by comparing 1) between control rat fed with ND (NN) and control rat fed with HFD (NH); 2) between diabetic rat fed with ND (DN) and diabetes rat with HFD (DH); 3) between diabetic rat fed with ND treated with metformin (DNM) and diabetic rat fed with HFD treated with metformin (DHM). R2Y is the fraction of variance of Y matric while Q2Y is the predictive accuracy of the model, both these values are used in OPLS_DA analysis. OPLS-DA is a useful statistical analysis tool to generate useable information from a challenging and non-systematic data, when a

Table 2. Group comparison: the sensitivity, specificity, accuracy, R2Y, and Q2Y of OPLDS-DA models.

Group comparison	Sensitivity,%	Specificity,%	Accuracy,%	R2Y	Q2Y
High Fat Diet					
NN versus NH	100	100	100	0.832	0.455
DN versus DH	83.33	100	91.67	0.760	0.401
DNM versus DHM	85.71	100	92.31	0.700	0.483
Diabetes					
NN versus DN	80	100	90.91	0.86	0.747
NH versus DH	100	100	100	0.97	0.397
Metformin					
DN versus DNM	100	83.33	91.67	0.967	0.677
DH versus DHM	100	71.43	86.67	0.575	0.42

NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat fed with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin). R2Y (the fraction of variance of Y matric); Q2Y (the predictive accuracy of the model).
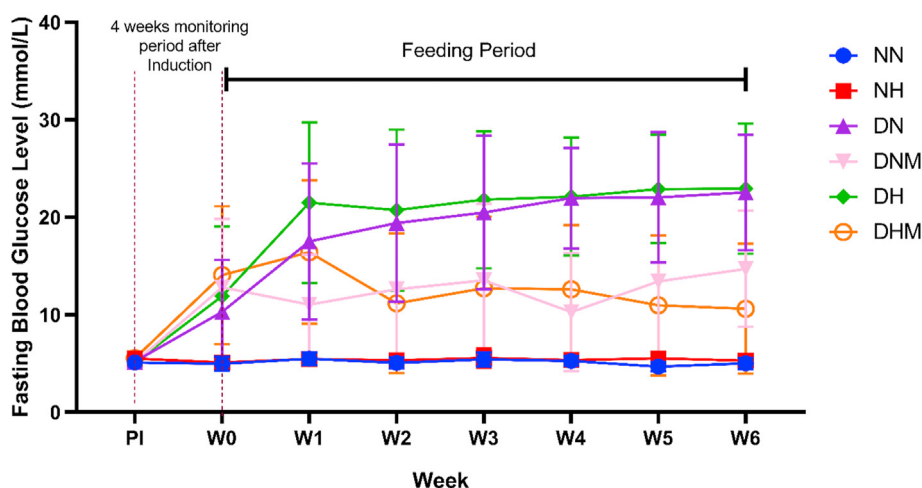

Figure 1. Fasting blood glucose of the rats throughout the six weeks of experiment period. (mean ± SD) PI is pre-induction to diabetes condition, W0 is the starting point of feeding program. NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat fed with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin).

separation was generated between data contributors from two comparison groups, this indicated that the two groups are of significant different statistically based on the data contributors [28]. The OPLS-DA showed clear separation for all three comparisons (Figure 2A–C). All the comparisons had sensitivity, specificity, and accuracy above 83 % (Table 2). The metabolites that contributed to the separation (VIP>1) were identified and the identities of the compounds are listed in Table 3. The compounds labelled as unknown were the metabolites that cannot be identified using Biological Magnetic Resonance Bank and the other two databases. In the comparison between ND-fed and HFD-fed of control rats, HFD caused the increase of citrate, sarcosine, and phenylacetylglycine while HFD decreased hippurate, trimethylamine-N-oxide (TMAO), 2-oxoglutarate, creatinine, and taurine. As for the comparison between ND-fed and HFD-fed of diabetic rats; acetoacetate, citrate, creatinine, allantoin, acetone, oxoglutarate and 3-hydroxybutyrate were increased by HFD. Lastly, for the comparison between the metformin treated groups of ND-fed and HFD-fed of diabetic rats; citrate, allantoin, methylamine, sarcosine, methylguanidine, and creatinine were increased by HFD while lactate was decreased by HFD.

3.3. The effect of diabetes on urinary metabolites

The effect of diabetes on rats was evaluated by comparing between 1) control rat fed with ND (NN) and diabetic rat fed with ND (DN); 2) between control rat fed with HFD (NH) and diabetes rat with HFD (DH). Both comparisons showed clear separation in OPLS-DA analysis (Figure 2D and E). The sensitivity, specificity and accuracy level for both comparisons were above 80 % (Table 2). The metabolites that contributed to the separation (VIP>1) were identified and listed in Table 4. The metabolites that showed reduction in intensity due to diabetes, regardless of the type of diet fed were hippurate, citrate, allantoin, creatinine, succinate, sarcosine and 2-oxoglutarate. Lactate was reduced in diabetic rats fed with ND. On the other hand, cis-aconitate, penylacetylglycine, and acetyglycine were decreased in diabetic rats fed with HFD.

3.4. The effect of metformin on urinary metabolites of diabetic rats

The effect of metformin treatment was evaluated by comparing 1) between diabetic rat fed with ND (DN) and diabetic rat fed with ND treated with metformin (DNM); 2) between diabetic rat fed with HFD (DH) and diabetic rat fed with HFD treated with metformin (DHM). The

metformin region was excluded in the OPLS-DA analysis as intact metformin was excreted in urine after the treatment [29]. Both comparisons showed clear separation in OPLS-DA analysis (Figure 2F and G). The sensitivity, specificity, and accuracy levels for both comparisons were above 71 % (Table 2). The metabolites that contributed to the separation (VIP>1) were identified and listed in Table 5. In the diabetic rats fed with ND, metformin caused the increase of lactate and 3-hydroxybutyrate. On the other hand, lactate, allantoin, citrate, succinate, sarcosine and creatinine were increased by metformin in diabetic rats fed with HFD. Metformin also caused the decrease of 3-hydroxybutyrate in diabetic rats fed with HFD.

4. Discussion

Urine has good diagnostic values, because it is not involved in body homeostasis [14, 15]. Thus, the analysis of urinary metabolites provides useful information on physiological changes of the body. HFD is defined as diet that contained >30 % fat [3]. According to this definition, many of our daily food intakes are consisting of HFD. Vegetable oil is a preferred fat used in food preparation, this is due to health concern with the perception that animal fat is a bad choice for health [30]. Therefore, this study was designed to understand the physiological impact of palm oil-enriched HFD on health through NMR analysis of urinary metabolites. In addition, metformin was given to diabetes rats and the metabolites profiles were evaluated. Urea, the most abundant metabolite in urine, was excluded from the analysis due to its potential interference with water suppression effect by 1H-NMR [31].

The urinary metabolites profiles have shown that feeding of palm oil-enriched HFD caused metabolites changes to diabetes rats. In general, most of these metabolites were the components of known metabolic pathways, although most of these were the catabolism processes, the data may be biased as only monomolecular organic compounds were captured in the analysis.

The altered levels of metabolites detected showed a high possibility that tricarbocylic acid cycle (TCA) was affected. Figure 3 shows TCA cycle, and the metabolites identified in this study with altered levels of excretion in the urine are as labelled.

TCA cycle is the key metabolic pathway involved in various metabolic networks, including carbohydrates, fat, and protein metabolisms. TCA cycle feeds NADH into oxidative phosphorylation pathway to produce energy required by body in the form of ATP [33]. A parallel decreased in
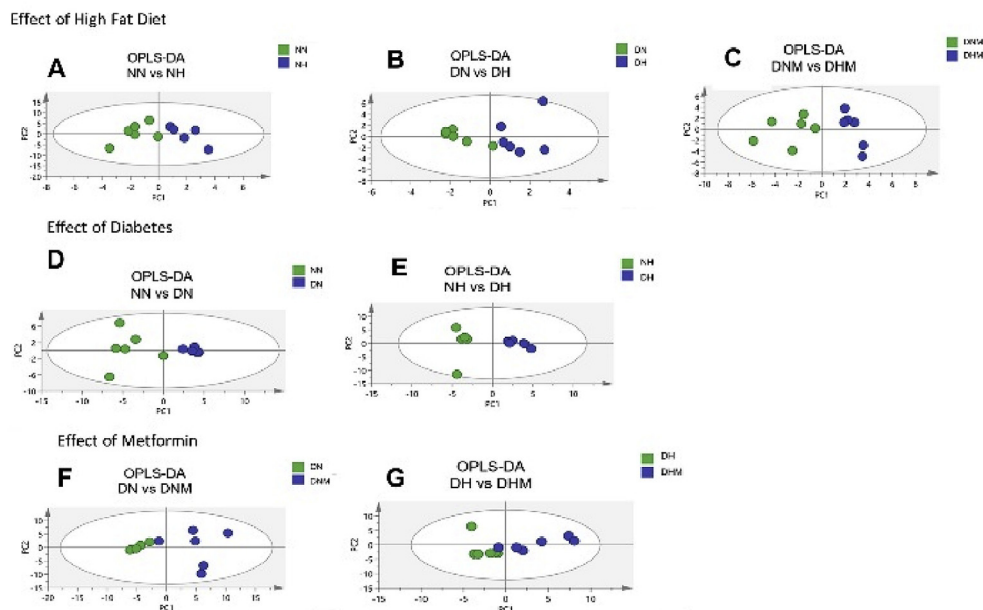

Figure 2. OPLS-DA score scatter plots of all the rat's comparison models. Panel A: OPLS_DA of NN vs NH; Panel B: OPLS-DA of DN vs DH; Panela C: OPLS-DA of DNM vs DHM; Panel D; OPLS-DA of NN vs DN; Panel E: OPLS-DA of NH vs DH; Panel F: OPLS-DA of DN vs DNM; Panel G: OPLS-DA of DH vs DHM. NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat fed with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin).

Table 3. Metabolites that were altered by HFD.

ND fed control rats versus HFD fed control rats

Compound	Chemical shift, ppm	Relative Intensity NN	Relative Intensity NH
Hippurate	7.8226–7.8542 (d)	3.531 ± 1.738	3.265 ± 1.555
	7.538–7.581 (t)	3.332 ± 1.671	3.053 ± 1.347
Citrate	2.5277–2.5681 (d)	4.603 ± 2.906	5.295 ± 2.566
	2.6689–2.713 (d)	2.848 ± 1.693	3.179 ± 1.698
TMAO	3.264–3.27 (s)	1.209 ± 0.545	0.686 ± 0.231
Sarcosine	2.722–2.734 (s)	1.001 ± 0.395	1.198 ± 0.497
	3.583–3.593 (s)	0.786 ± 0.289	1.257 ± 0.596
2-oxoglutarate	2.4306–2.466 (t)	1.347 ± 0.661	1.212 ± 0.877
Creatinine	3.035–3.052 (s)	9.758 ± 4.004	6.859 ± 2.363
	4.048–4.057 (s)	6.927 ± 3.017	4.709 ± 1.656
Phenylacetylglycine	3.676–3.683 (s)	1.027 ± 0.524	1.515 ± 0.392
	3.75–3.761 (d)	1.936 ± 0.636	2.415 ± 0.767
Taurine	3.4149–3.4453 (t)	0.676 ± 0.317	0.362 ± 0.109
Unknown	3.593–3.601 (s)	1.121 ± 0.527	0.632 ± 0.222
Unknown	3.642–3.653 (s)	0.976 ± 0.255	1.506 ± 0.939
Unknown	3.654–3.66 (s)	0.839 ± 0.346	1.390 ± 0.731
Unknown	3.669–3.674 (s)	0.524 ± 0.207	0.820 ± 0.333
Unknown	3.701–3.711 (s)	1.250 ± 0.490	0.798 ± 0.066
Unknown	3.721–3.728 (s)	0.785 ± 0.255	1.339 ± 0.526
Unknown	3.815–3.827 (s)	1.438 ± 0.483	1.727 ± 0.697

ND fed diabetic rats versus HFD fed diabetic rats

Compound	Chemical shift, ppm	Relative Intensity DN	Relative Intensity DH
Acetoacetate	2.282–2.288 (s)	0.055 ± 0.026	1.859 ± 4.198
Citrate	2.519–2.567 (d)	1.820 ± 0.649	3.479 ± 1.660
	2.688–2.715 (d)	1.707 ± 0.639	3.110 ± 1.346
Creatinine	3.043–3.053 (s)	0.7440 ± 0,439	1.407 ± 0.448
	4.058–4.065 (s)	0.630 ± 0.284	1.084 ± 0.354
Allantoin	5.388–5.41 (s)	1.214 ± 0.404	1.674 ± 0.411
Acetone	2.234–2.244 (s)	0.154 ± 0.066	0.558 ± 0.888
2-oxoglutarate	2.426–2.467 (t)	1.440 ± 0.619	1.877 ± 1.110
3-hydroxybutyrate	2.298–2.306 (q)	0.083 ± 0.036	0.350 ± 0.381

ND fed diabetic rats versus HFD fed diabetic rats with metformin treatment

Compound	Chemical shift, ppm	Relative Intensity DNM	Relative Intensity DHM
Citrate	2.526–2.572 (d)	1.450 ± 0.921	3.097 ± 1.582
	2.678–2.722 (d)	0.918 ± 0.566	1.783 ± 0.882
Allantoin	5.388–5.409 (s)	0.276 ± 0.058	0.289 ± 0.052
	6.007–6.054 (s)	0.709 ± .216	1.332 ± 0.621
Methylamine	2.607–2.613 (s)	0.120 ± 0.048	0.236 ± 0.109
Sarcosine	2.723–2.728 (s)	0.410 ± 0.101	0.789 ± 0.283
Methylguanidine	2.825–2.848 (s)	0.289 ± 0.058	0.598 ± 0.185
Creatinine	4.056–4.065 (s)	1.065 ± 0.197	2.131 ± 0.930
Lactate	1.322–1.348 (d)	15.165 ± 14.421	2.577 ± 3.866
	4.100–4.149 (q)	7.713 ± 7.082	1.717 ± 2.216

NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin).
s, singlet; d, doublet; t, triplet; q, quartet.

Table 4. Metabolites that were altered by diabetes condition.

Control rat fed on ND versus diabetic rat fed on ND

Compound	Chemical shift, ppm	Relative Intensity NN	Relative Intensity DN
Hippurate	7.536–7.564 (t)	3.045 ± 1.867	0.628 ± 0.336
	7.622–7.665 (t)	1.533 ± 0.919	0.258 ± 0.180
	7.823–7.86 (d)	3.402 ± 2.004	0.580 ± 0.410
Citrate	2.529–2.568 (d)	7.066 ± 4.984	1.749 ± 0.565
	2.66302.713 (d)	6.875 ± 4.495	1.770 ± 0.634
Lactate	1.327–1.346 (d)	1.475 ± 2.126	0.508 ± 0.502
Allantoin	5.411–5.42 (s)	3.150 ± 0.955	1.168 ± 0.386
	6.016–6.052 (s)	0.926 ± 0.419	0.264 ± 0.140
Creatinine	3.037–3.052 (s)	9.055 ± 4.531	0.767 ± 0.417
	4.049–4.058 (s)	4.387 ± 2.379	0.611 ± 0.282
Succinate	2.403–2.414 (s)	1.530 ± 0.689	0.440 ± 0.228
Sarcosine	2.723–2.731 (s)	0.867 ± 0.425	0.176 ± 0.066
2-oxoglutarate	2.429–2.466 (t)	2.970 ± 1.250	1.452 ± 0.650

Control rat fed on HFD versus diabetic rat fed on HFD

Compound	Chemical shift, ppm	Relative Intensity NH	Relative Intensity DH
Creatinine	3.036–3.049 (s)	8.244 ± 4.993	1.382 ± 0.430
	4.046–4.058 (s)	5.837 ± 3.685	1.043 ± 0.271
Citrate	2.519–2.566 (d)	4.010 ± 2.648	2.157 ± 0.983
	2.667–2.707 (d)	4.077 ± 2.964	2.004 ± 0.842
Allantoin	5.385–5.404 (s)	3.453 ± 1.704	1.553 ± 0.350
Phenylacetylglycine	7.344–7.384 (t)	0.174 ± 0.106	0.052 ± 0.018
Hippurate	7.535–7.578 (t)	2.478 ± 1.633	0.821 ± 0.276
	7.824–7.851 (d)	2.645 ± 1.796	0.842 ± 0.312
Sarcosine	2.721–2.728 (s)	0.992 ± 0.670	0.316 ± 0.094
Cis-aconitate	5.688–5.697 (t)	0.984 ± 0.710	0.390 ± 0.114
Succinate	2.401–2.415 (s)	2.672 ± 2.503	0.594 ± 0.224
Acetylglycine	1.980–1.991 (s)	0.593 ± 0.382	0.172 ± 0.032
2-oxoglutarate	2.427–2.464 (t)	2.523 ± 1.819	2.316 ± 0.899
Unknown	1.198–1.203 (s)	0.125 ± 0.064	1.964 ± 4.670
Unknown	1.216–1.223 (s)	0.163 ± 0.103	1.974 ± 4.710
Unknown	2.034–2.044 (s)	0.877 ± 0.737	0.304 ± 0.112
Unknown	2.278–2.284 (s)	0.224 ± 0.141	1.892 ± 4.275

NN (control rat fed with ND); NH (control rat fed with HFD); DN (diabetic rat fed with ND).
s, singlet; d, doublet; t, triplet.

2-oxoglutarate were detected, this data may indicate that besides TCA cycle, the metabolites were the results of other pathways.

2-oxoglutarate (2-OG) or α-ketoglutarate is the essential nitrogen transporter in the metabolic pathways. 2-OG is generated from isocitrate and decarboxylated into succinyl-CoA in TCA cycle. Urinary 2-OG level was reduced in diabetic rats and in diabetic rats fed with HFD. In contrary, 2-OG levels were increased in control rats fed with HFD. These contradicting effects of HFD on 2-OG levels in control rats and diabetic rats may be attributed to the diabetes condition itself that lowered the 2-OG levels [34].

Urinary lactate was found lower in diabetic rats and diabetic rats fed with HFD, while metformin treatment increased urinary lactate levels. Diabetes reduces lactate production [35, 36]. Feeding of HFD may divert energy metabolism from glucose consumption to fatty acids consumption and caused a fall in urinary lactate by HFD. Metformin's role in improving glucose homeostasis in body may alter energy metabolism, in which we detected increased level of lactate in diabetic rats treated with metformin.

Fatty acid β-oxidation and ketogenesis may possibly enhance by HFD under diabetic condition. This was revealed by the increased levels of

citrate, cis-aconitate, 2-oxoglutarate, and succinate levels were identified in diabetic rats, which may imply the reduction of TCA cycle. On the other hand, metformin treatment on diabetic rats may reinstate the TCA cycle, where increased levels of citrate and succinate were detected in rats' urine. The effect of HFD on TCA cycle of diabetes rats was not certain; where an increase level of citrate and a decrease level of

Table 5. Metabolites that were altered by metformin treatment on diabetes rats. DN (diabetic rat fed with ND).

Diabetic rats fed on ND with metformin treatment versus Diabetic rats fed on ND without metformin treatment

Compound	Chemical shift, ppm	Relative Intensity DN	Relative Intensity DNM
Lactate	1.323–1.344 (d)	0.508 ± 0.511	29.786 ± 28.886
	4.100–4.1492 (q)	0.424 ± 0.167	8.503 ± 7.712
3-hydroxybutyrate	1.194–1.213 (d)	0.049 ± 0.014	2.799 ± 2.518

Diabetic rats fed on HFD with metformin treatment versus Diabetic rats fed on HFD without metformin treatment

Compound	Chemical shift, ppm	Relative Intensity DH	Relative Intensity DHM
Lactate	1.319–1.348 (d)	0.294 ± 0.0.99	5.089 ± 7.722
Allantoin	5.39–5.409 (s)	1.507 ± 0.470	3.606 ± 1.354
	6.012–6.071 (s)	0.389 ± 0.141	1.363 ± 0.682
3-hydroxybutyrate	1.193–1.212 (d)	7.639 ± 13.981	1.553 ± 1.552
	2.317–2.405 (q)	2.571 ± 4.336	1.045 ± 0.611
	4.135–4.193 (s)	1.518 ± 2.557	0.688 ± 0.384
Citrate	2.528–2.586 (d)	2.699 ± 1.535	4.924 ± 2.428
	2.679–2.720 (d)	2.538 ± 1.497	4.652 ± 2.262
Succinate	2.407–2.413 (s)	0.408 ± 0.175	0.899 ± 0.484
Sarcosine	2.722–2.726 (s)	0.265 ± 0.113	0.804 ± 0.320
Creatinine	4.053–4.066 (s)	0.877 ± 0.371	2.157 ± 0.865

DN (diabetic rat fed with ND); DNM (Diabetic rat fed with ND treated with metformin); DH (diabetic rat fed with HFD); DHM (diabetic rat fed with HFD treated with metformin)
s, singlet; d, doublet; q, quartet.

ketone bodies (acetoacetate and acetone and 3-hydroxybutyarate) in diabetic rats fed with HFD. Under the condition of high fatty acid levels, ketogenesis will be a preferred pathway as TCA cycle capacity is overwhelmed [37]. Increased levels of ketone bodies can be an indication that HFD induced a greater stress on diabetes condition compared to ND. This is because these ketone bodies are induced under oxidative stress [38].

Taurine level was found decreased in control rats fed with HFD. Taurine is the major metabolite in bile acid metabolism. Taurine involves in cholesterol and bile salts conjugation, osmoregulation, antioxidation, ion movement, signalling modulation, membrane stabilisation, and neurotransmitters modulation [39]. Taurine is a by-product of the cysteine sulfonic acid pathway [40], that was suppressed in HFD induced obese mice [41].

Gut microbiome plays a significant role in human health as it is involved in various metabolic functions. Gut microbes produce biologically active metabolites through its energy harvesting and storage ability [42]. HFD and diabetes were associated with a change of gut bacterial composition, which can be recognised by specific metabolites [43, 44]. In this study, HFD fed control rats showed an increase in urinary phenylacetylglycine and a decrease in hippurate. In contrary, diabetic rats showed a decrease of phenylacetylglycine, acetylglycine and also hippurate. These results suggested that HFD may alter gut microbiome profiles in the rats. Hippurate level is associated with diet intake as it is a sensitive indicator for diet [45] and lower level of hippurate was reported in type 2 diabetes patients [46]. Acetylglycine is a product of pheylacetylglycine, while pheylacetylglycine is a biomarker for phospholipid accumulation [47], which is also associated with fat content in the body.

Choline is an important nutrient for cellular structure integrity, methyl metabolism and the transport/metabolism of lipid in the body [48, 49] and choline metabolism is strongly associated with gut microbiota activity. Trimethylamine-N-oxide (TMAO), methylamine and sarcosine are the products of choline metabolism [50]. In this study, urinary TMAO level was lower in control rats fed with HFD compared to those fed

with ND, which may suggest the suppression of choline metabolism by HFD. Metformin treatment on HFD-fed diabetic rats showed an increase levels of methylamine and sarcosine, which indicate metformin treatment reverts choline metabolism by HFD.

Creatinine is the breakdown/waste product of creatine from muscle and protein metabolism. In this study, urinary creatinine level was lower in control rats fed with HFD compared to its counterpart on ND. It was also lower in diabetic rats compared with control rats. In contrary, creatinine levels was higher in both diabetic rats fed with HFD with/ without metformin treatment compared to those fed with ND, and in metformin treated diabetic rats fed with HFD compared to its counterpart without metformin treatment. HFD increases urinary creatinine excretion [51, 52, 53]. Increased level of creatinine indicates renal dysfunction [54], a condition when diabetes progressed and injured the kidney. This study was carried out at the early phase of diabetes, the low level of creatinine in diabetic rats indicated that kidney injury has not occurred at this early phase of the disease. Therefore, the increased level of creatinine observed in this study may solely due to the intake of HFD. Methylguanidine is formed due to the oxidation of creatinine [55, 56, 57]. In this study, we see a parallel level of methylguanidine and creatinine, where urinary level of methylguanidine was higher in diabetic rats fed with HFD treated with metformin than its counterpart fed on ND. Methylguanidine is a uremic toxin that accumulates in the body fluid of uremic patients or kidney failure patients [58].

Allantoin is generated from non-enzymatic oxidation of uric acid with ROS [59]. It is a biomarker of oxidative stress [60]. Plasma allantoin is higher in people with diseases related to oxidative stress such as rheumatoid arthritis, diabetes, and stroke [61, 62, 63, 64]. Urinary allantoin excretion also accurately reflects GFR as it is not reabsorbed in the proximal tubule of kidneys [65]. Therefore, oxidative stress and kidney function (GFR) will affect the allantoin level in urine. In this study, urinary allantoin level was higher in HFD diabetic rats with/without metformin treatment compared to their counterparts fed with ND, and higher in HFD-fed diabetic rat with metformin treatment compared to its counterpart without metformin treatment. In contrary, the level was lower in diabetic rats compared to control rats. HFD and diabetes increase ROS and oxidative stress [66, 67]. The association of allantoin with HFD implies HFD may induce stress to the animals, both diabetic and healthy at the early phase of HFD consumption.
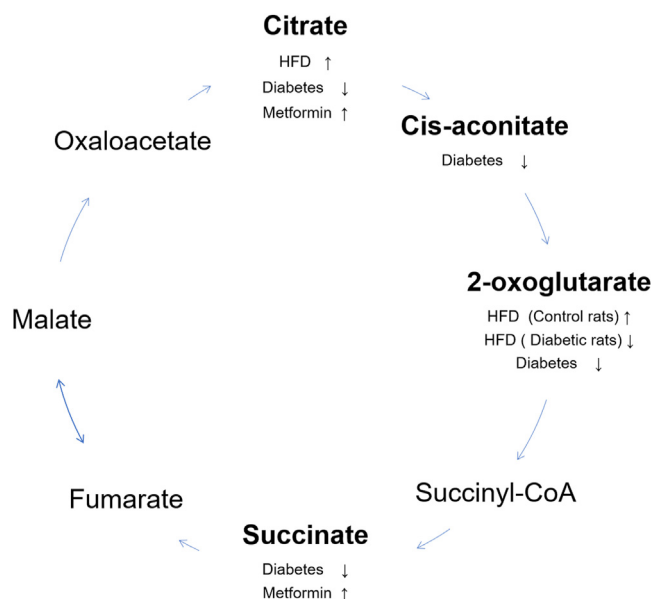
Figure 3. TCA cycle and its metabolites (adapted from Ryan et al. (2019) [32]).

5. Conclusion

In this study, we aimed to investigate the impact of palm oil-enriched HFD consumption on diabetes condition at the early phase of the disease. The data obtained revealed that continuous consumption of HFD for a short period has adversely altered the metabolic pathways of the rats, both healthy and diabetic. The metabolites profiles of healthy and diabetic rats indicated that palm oil-enriched HFD induced oxidative stress to the animals. This effect was more prominent in the diabetes rats, where the increased excretion of creatinine and allontoin has marked the early kidney injury in diabetic rats. On the other hand, metformin treatment combined with normal diet lessened the physiological stress caused by diabetes disease. It is noteworthy that this current study is at preliminary stage, and the interpretation of the data is limited to the known metabolism pathways. Therefore, we acknowledge the possibility that the metabolites may be the products of other metabolism sources. Future study on the long-term effect of palm oil-enriched HFD on insulin sensitivity is worth investigating.

Declarations

Author contribution statement

Xuan-Yi Sim: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Baharudin Ibrahim: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lay-Harn Gam: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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