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Original Article

Lipopolysaccharide as a biomarker of gut dysbiosis in diabetic Wistar rats – A pre-clinical experimental study

Sowmiya Thiyagarajan[1], Emmanuel Bhaskar[2], Veeraraghavan Gayathri[3], Parthasarathy Mohanalakshmi[4], Santhi Silambanan[1]

Departments of [1]Biochemistry, [2]General Medicine and [3]Centre for Toxicology and Developmental Research (CEFTE), Sri Ramachandra Institute of Higher Education and Research, [4]Department of Biochemistry, Sri Muthukumaran Medical College Hospital and Research Institute, Chennai, Tamil Nadu, India.

***Corresponding author:**
Santhi Silambanan,
Department of Biochemistry,
Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India.

santhisilambanan@sriramachandra.edu.in

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ABSTRACT

Objectives: The increasing trend of type 2 diabetes mellitus (T2DM) poses a global health concern. The features of T2DM include hyperglycaemia and dysfunctional insulin secretion or action levels. Recent research indicates that the gut microbiota profile gets modified in diabetes. This is followed by an incremental increase in serum lipopolysaccharide (LPS) levels, which is found to have a bearing on the pathology of diabetes. LPS is a constituent of the outer wall of bacteria, particularly the Gram-negative ones. Modified gut microorganisms may interfere with intestinal barrier integrity and function, as well as host metabolic and signalling pathways. The study aimed to assess the levels of LPS as a biomarker for gut dysbiosis in diabetic adult Wistar rats.

Materials and Methods: This pre-clinical, experimental study was conducted at CEFTE within the premises of the Sri Ramachandra Institute of Higher Education and Research. Institutional animal ethics committee approval was obtained. Adult male Wistar rats, 12 in number, were grouped as healthy controls (six rats) and diabetic cases (six rats). Diabetes was brought about in the case group with a high-fat diet followed by a streptozotocin injection. Plasma glucose, total cholesterol, triglycerides, insulin, homeostatic model assessment of insulin resistance (HOMA-IR) and LPS levels were analysed. Statistical significance was considered as $P \leq 0.05$.

Results: Research revealed that glucose, insulin, HOMA-IR, total cholesterol and triglyceride were found to be significantly increased in diabetic rats compared to healthy rats. In a comparison of diabetic rats with non-diabetic healthy rats, there was a statistically significant increase in LPS in diabetic rats.

Conclusion: Elevated LPS levels in diabetic rats suggest increased gut permeability and systemic inflammation, contributing to insulin resistance and the progression of T2DM. The study could highlight that LPS may serve as a potential biomarker for gut dysbiosis in diabetes. Further research may help explore the various molecular pathways concerned with this type of association and the various targeted therapies in handling gut dysbiosis in diabetes.

Keywords: Diabetes mellitus, Gut dysbiosis, Gut integrity, Insulin resistance, Lipopolysaccharide

INTRODUCTION

In 2021, the International Diabetes Federation declared that around 536.6 million diabetics are present worldwide between 20 and 79 years of age. This is believed to amount to at least 10% of the global population, and by 2045, there may be an exponential rise in diabetics to 783.2 million.[1] Globally, diabetes mellitus is responsible for 10% of deaths, and with regard to disability-adjusted

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life years, type 2 diabetes mellitus (T2DM) is found to rank as the 14th leading cause.[2] In addition to T2DM, there are around 47 million with borderline hyperglycaemia, which can further increase the risk of developing T2DM.[1] The prevalence of T2DM poses a significant challenge in India and China, where the incidence rates are the highest due to their larger populations. Amongst the countries across the globe, China holds the highest diabetic population, reaching 140 million in 2021, followed by India with 74 million cases of diabetes mellitus.[1] In India, approximately one in six individuals suffers from diabetes mellitus, with 49.7% being underdiagnosed, which represents nearly half of all cases.[3]

Diabetes mellitus is associated with macrovascular and microvascular problems.[4] Emerging studies suggest a potential link between diabetes mellitus and gut dysbiosis.[5] In gut dysbiosis, the profile of microorganisms is altered so that the beneficial ones are much less than the harmful ones. The harmful microorganisms pave the way for various pathological changes in the human intestine. This disturbance in the gut microorganisms is found to be connected with insulin resistance (IR) in T2DM.[6] The analysis of gut dysbiosis is by culturing the microbiota followed by gene sequencing. These methods assess the microbial diversity, specific bacterial taxa and the overall composition of the gut microbiome. However, these approaches are often deemed to be costly, cumbersome and time-consuming.[7] Normally, lipopolysaccharide (LPS) is produced by Gram-negative bacteria colonising the gastrointestinal tract. In healthy individuals, the colonies of Gram-negative bacteria are much smaller than those of Gram-positive bacteria. In states of altered gut bacteria profile found in diseases, Gram-negative bacteria tend to become more and control normal commensal Gram-positive bacteria. LPS and other proteins play a major role in altering gut permeability.[8] This altered gut permeability enhances the spread of this endotoxin to other organs such as the liver, pancreas, adipose tissue and skeletal muscle. Since Gram-negative bacteria are mainly involved in the production of endotoxin-LPS, there is found to be an association between gut dysbiosis, LPS and diabetes mellitus. Measuring LPS levels in the bloodstream offers a more accessible and cost-effective alternative. LPS levels provide valuable insights into the systemic impact of dysbiosis.

The anatomical features of rodents match that of humans, even though it is not a complete similarity.[9] The specific composition of gut microbiota may differ between humans and rats at the phyla level, but the balance of Gram-negative and Gram-positive bacteria is similar. The hypothesis that gut inflammation can lead to increased intestinal permeability, also known as leaky gut, allowing LPS to translocate into the bloodstream, has been supported by various studies.[10] Chronic low-grade gut inflammation and an altered gut microbiome are believed to disrupt tight junction proteins within the intestinal epithelium, which normally prevent the passage of endotoxins like LPS into the systemic circulation.[11] The underlying mechanisms of gut dysbiosis involve alterations in the ratio of Gram-negative and Gram-positive bacteria, similar in humans and rats. During gut dysbiosis, the balance between these bacterial groups is disrupted, resulting in an increase in Gram-negative bacteria, the primary source of LPSs. This study aimed to measure alterations in serum LPS levels in diabetic male Wistar rats.

MATERIALS AND METHODS

Study design, sample size and grouping

The preclinical experimental study was performed at the Centre for Toxicology and Developmental Research (CEFTE), Sri Ramachandra Institute of Higher Education and Research, Chennai, India.

Adult male Wistar rats of 12 in number were 8–10 weeks old and weighed between 180 and 220 g. The rats were grouped into two. The number of rats included in the research was decided according to the Federer calculation.[12]

1. Group 1: Healthy control (n=6)
2. Group 2: Diabetic case (n=6).

Inclusion criteria

- Animals are deemed healthy without any pre-existing medical conditions.

Exclusion criteria

- Adult female Wistar rats were excluded. Female rats undergo varied hormonal alterations, especially during the fertility cycles, which may have an influence on the levels of biomarkers.

Care of animals during the study period:

The adult male Wistar rats were accommodated in polypropylene cages containing three animals. Bedding in the cages consisted of paddy husks, which were changed periodically. The cage conditions were conducive to the animals - temperature (19–23°C), humidity (30–70%) and air changes (12–15/h). Animals had artificial light and darkness for 12 hours each. The animals had free access to water and feed. Figure 1 shows the scheme included in the study.

Utilising a high-fat diet (HFD) alone may not reliably induce an optimal diabetic model; hence, HFD was combined with a low dose of streptozotocin (STZ) in the present study. HFD alone is known to cause IR and metabolic disturbances, but its ability to consistently mimic the pathophysiology of T2DM is

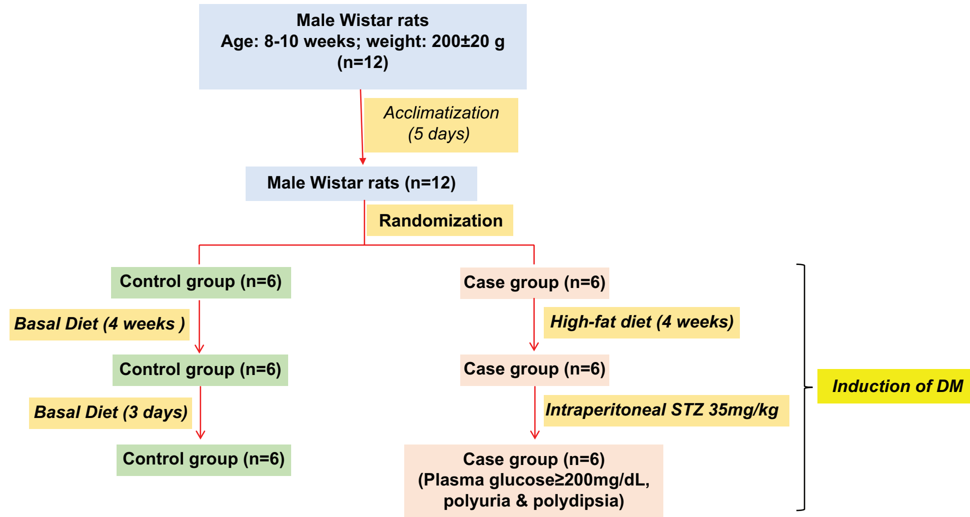

Figure 1: Methodology adopted for induction of diabetes mellitus in adult male Wistar rats. STZ: Streptozotocin; DM: Diabetes mellitus.

limited.[13] STZ is highly effective at damaging pancreatic beta cells and inducing hyperglycaemia. To balance these factors, the combination of HFD and a low dose of STZ was adopted to create a model closely resembling the progression of T2DM, characterised by IR and partial beta-cell dysfunction.

After being received in the research centre, the animals were allowed to acclimate for a week. All the animals were on a basal diet, and in the next week, the animals were randomised and caged into four units, with three in each cage. The weight, feed, water and calorie intake were measured. Six rats were included as healthy controls and fed a basal diet for 4 weeks. In the other two cages, rats were grouped as cases (group 2) in which diabetes mellitus was induced using an HFD for 4 weeks. The nutrient composition of the HFD included fat, carbohydrates and proteins in the proportion of 40, 43 and 17%, respectively, along with adequate quantities of micronutrients.[14] After giving a HFD for 4 weeks, a 35 mg/kg intraperitoneal injection of STZ was given subsequent to 6 h of fasting.[15] Three days after STZ injection, the animals were considered to be diabetic and showed a blood glucose minimum of 200mg/dL along with clinical features such as polyuria as well as polyphagia.

Sample collection and analysis

Blood samples were taken by trained personnel involved in the routine care of the animals. The researcher (ST) was present during the sample collection. The rats were fed the usual diet, and food and water were provided *ad libitum*. The rats were on alternating 12 hours of light and darkness. In the mornings, a serum sample for LPS was collected. After the sample had clotted, the serum was separated and stored at −80°C until analysis. Stable for approximately 1 month at 2–8°C. Frozen aliquots can be stored for up to 2 years. Repeated freeze/thaw cycles are not recommended.[16]

There are several steps to control for potential confounders in our study, including the rats' stress levels, handling differences and food intake variability. To minimise stress, we standardised handling procedures, ensuring that all rats were handled by the same trained personnel, and we acclimatised the animals to adjust to the new environment before the study. This approach helped to reduce the variability in animal handling techniques and stress responses. In addition, we maintained a consistent routine of interactions with the animals, including the timing and duration of handling, to ensure uniform treatment throughout the study. Regarding food intake variability, we provided a standardised diet and monitored daily food consumption for each rat, allowing us to account for any differences in food intake during our analyses.

From all the animals, blood samples were obtained from the retroorbital vein of the eye, and plasma and serum were stored at −80°C. Glucose, total cholesterol and triglyceride were analysed by standard methods. LPS and insulin were analysed by enzyme-linked immunosorbent assay (ELISA). The LPS ELISA kit was sourced from Elabscience and stored at 2–8°C to maintain stability. The method employed a sandwich ELISA technique. LPS in the samples binds to pre-coated antibodies on the plate, followed by the addition of a biotinylated LPS antibody and Streptavidin-horse-radish peroxidase (HRP). The reaction was detected colorimetrically at 450 nm. The assay had a standard curve ranging from 2 to 600 endotoxin units (EU)/L, with a 0.93 EU/L sensitivity. The precision was high, with an intra-assay coefficient of variation (CV) of <8% and an inter-assay CV of <10%.

LPS molecule consists of various components with additional lipids and carbohydrates. This composition tends to change with pre-analytical procedures used to prepare the samples.

Since the serum was separated using the same protocol for all the samples, variations in the LPS molecules could not have occurred. With regard to the type of LPS from different Gram-negative bacteria, they are different in structure across the species. However, the mode of action, i.e. binding to its receptor and initiating inflammation, is the same for almost for all the LPS-producing Gram-negative bacteria.

To analyse gut dysbiosis, 16 S ribonucleic acid (RNA) is measured in the faecal samples. We could compare the biomarkers with 16S RNA during the initial scrutiny meeting. However, the experts suggested that the measurement of 16S RNA is labour-intensive and not cost-effective. Hence, we modified the research proposal. However, we have decided to continue to the next stage before we intend to carry on human beings.[7]

Statistical analysis

Data were analysed in the Statistical Package for the Social Sciences software (version 16). Student '*t*'-test was applied to compare the mean and standard deviation of the groups. Statistical significance was fixed as $P \leq 0.05$.

RESULTS

The body weight, feed intake, water intake, calorie intake, plasma glucose, total cholesterol, triglyceride, insulin, homeostatic model assessment of IR (HOMA-IR) and serum LPS of all the animals were analysed and subjected to statistical analysis and shown in Table 1.

Table 1: Plasma glucose and serum LPS levels in Group 1 (Healthy control) and Group 2 (Diabetic case).

Parameter	Group 1 healthy control (*n*=6)	Group 2 diabetic case (*n*=6)	*P*-value
Body weight (g)	272.97±77.38	292.87±43.74	0.31
Feed intake (g)	54.67±14.33	42.25±5.15	0.18
Water intake (mL)	67.29±18.79	53.64±3.54	0.20
Calorie intake (g)	164.01±42.98	199.82±24.37	0.206
Glucose (mg/dL)	140.17±16.53	520.83±55.95	<0.001**
Insulin (mIU/L)	4.59±0.86	7.41±1.09	<0.001**
HOMA-IR	1.58±0.33	9.51±1.65	<0.001**
Cholesterol (mg/dL)	57.17±11.05	106.17±59.92	0.03*
Triglyceride (mg/dL)	126.33±24.15	403.33±241.16	0.009*
LPS (EU/L)	52.64±15.82	160.48±50.51	<0.001**

HOMA-IR: Homeostatic model assessment of insulin resistance, LPS: Lipopolysaccharide, EU: Endotoxin units/mL (EU: Unit of measurement for endotoxin activity). Expressed in mean and SD, Student's t was used, **P*-value: Statistically significant; ***P*-value: Statistically highly significant.

There was no statistically significant difference between the groups in body weight, feed, water and calorie intake. LPS concentrations are expressed in EU; 10 EU/mL = 1.0 ng/mL.[17] Body weight, feed intake, water intake and calorie intake were not found to be significant in Group 1 and Group 2. The plasma glucose in Groups 1 and 2 was 140.17 ± 16.53 and 447.80 ± 141.78, respectively, with p<0.001. Insulin concentration in diabetic rats was 7.41 ± 1.09 mIU/L, significantly higher than in Group 1 (4.59 ± 0.86 mIU/L). In type 2 diabetes, the body initially attempts to adjust for IR by producing more insulin, leading to elevated insulin levels. HOMA-IR score was higher in Group 2 (9.51 ± 1.65) than in Group 1 (1.58 ± 0.33), demonstrating IR, a crucial aspect of type 2 diabetes. Cholesterol and triglyceride levels are significantly elevated in Group 2 with mean and standard deviation of 106.17 ± 59.92 and 403.33 ± 241.16 compared to Group 1 cholesterol levels (57.17 ± 11.05) and triglyceride (126.33 ± 24.15). The mean levels of LPS in Groups 1 and 2 were 52.64 ± 15.82 and 160.48 ± 50.51, respectively ($P < 0.001$) [Table 1].

Figure 2 shows the distribution of LPS levels in Group 1 (healthy control) and Group 2 (diabetic case).

DISCUSSION

The commensals colonising the gut include various microorganisms, such as bacteria, viruses, fungi and protozoa. There seems to be a complex relationship between the microorganisms and the host, and the microorganisms obtain energy by utilising nutrients from the food consumed by the host. Most microorganisms in the gut are bacteria, mostly belonging to *Firmicutes* and *Bacteroidetes*. These two phyla comprise approximately 60–80% and 20–30% of the overall gut microbiota. In addition, a few *Proteobacteria* and Actinobacteria are present in the gut. *Bacteroidetes*, a group of Gram-negative bacteria, is linked to higher gut endotoxin LPSs levels. During gut dysbiosis, *Bacteroidetes* outnumber *Firmicutes*, which is associated with high levels of LPS and inflammation.[18] Thus, the altered balance between the various bacteria in individuals

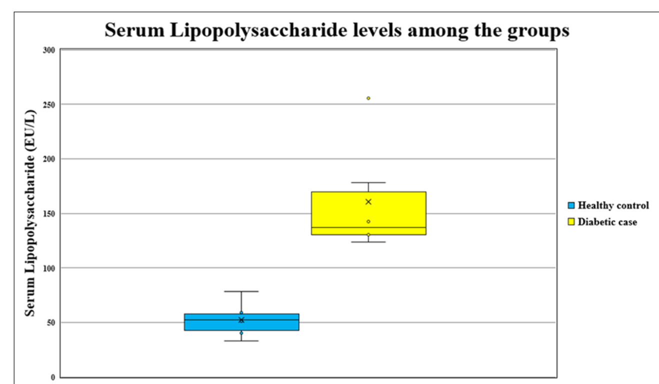

Figure 2: Box and whisker plots showing distribution of lipopolysaccharide amongst the groups.

with T2DM may cause the intestinal epithelial cells to become more permeable, thus allowing LPS to enter portal venous circulation and, thus, the systemic circulation. Moreover, LPS causes local intestinal inflammation, promoting IR and worsening glycaemic status in T2DM.[19]

In the present study, the plasma glucose level was much higher in diabetic rats than in healthy non-diabetic rats ($P < 0.001$). This showed that the induction of diabetes mellitus was effective in diabetic group 2 rats. The levels of insulin and HOMA-IR were higher in diabetic rats showing that the rats had IR compared to healthy rats. Total cholesterol and triglycerides were higher in diabetic rats, which showed that diabetics are prone to dyslipidaemia. Triglyceride showed much statistical significance compared to total cholesterol, reinforcing that higher triglyceride could aggravate IR in diabetics. The LPS levels in Groups 1 and 2 were 52.64 ± 15.82 and 160.48 ± 50.51 EU/L ($P < 0.001$) [Table 1 and Figure 2]. LPS is an important outer membrane component of the cell envelope of Gram-negative bacteria. When Gram-negative bacteria die, LPS is shed from the outer cell wall and excreted in faeces. Moreover, there is an elevated shedding of LPS in gut dysbiosis due to the increased ratio of LPS-producing bacteria.[20,21] LPS can potentially decrease the expression of tight junction proteins, thus leading to loss of integrity of the intestinal epithelium. This further disrupts intestinal integrity, leading to increased intestinal permeability. All these factors permit the entry of LPS from the intestinal lumen into the bloodstream.[22]

LPS is amphipathic in nature; it consists of the antigen O and the lipid A, along with an oligosaccharide chain. The core oligosaccharide is a conserved region connecting the O antigen with the lipid A. The O antigen is highly variable and can differ between different strains of bacteria.[23] Lipid A is mainly responsible for virulence as well as endotoxic effects.[24] Lipid A is recognised by a transmembrane protein called toll-like receptor 4, expressed on the surface of macrophages, monocytes, neutrophils and other immune cells. This binding activates intracellular signalling pathways, including the MyD88-dependent and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathways, thus magnifying the response.[25] As a result, even trace amounts of LPS in the blood can set off a series of events involving protein–protein interactions that increase the synthesis of proinflammatory cytokines such as tumour necrosis factor-alpha, interleukin-6 and interleukin-1β.[26] After binding to immune cells, LPS activates the mitogen-activated protein kinase signalling pathway. Thus, through various mechanisms, LPS sets the stage for IR in diabetes.[27] These cytokines can inhibit insulin signalling by inducing serine phosphorylation of insulin receptor substrate-1, thereby impairing the insulin receptor signalling cascade and contributing to IR.[28]

The present study suggested that there was increased gut permeability in diabetes mellitus, leading to the movement of LPS from the gut into the bloodstream, causing inflammation. Low-grade inflammation interferes with insulin action in the peripheral tissues, leading to hyperglycaemia and further organ damage due to hyperglycaemia. Hence, by supplementing with probiotics, gut microbiota may be returned to normal flora, improving diabetic status. Yes, this preliminary study showed results supporting our hypothesis; however, we plan to conduct further research involving multiple probiotic interventions. This future study will specifically investigate the potential of probiotics to reverse gut inflammation and glycaemic control associated with diabetes.[29]

Strengths

- The study found an association between altered microbiota in the gut and diabetes; thus, it gave new evidence for the pathogenesis of diabetes
- The use of well-defined experimental groups (healthy controls and diabetic rats) ensures clear comparative analysis
- Measuring serum LPS levels provides a cost-effective and accessible method for assessing gut dysbiosis.

Limitations

- The animal research was conducted solely on male Wistar rats and excluded female Wistar rats. We recognise the importance of studying both sexes for generalisability, as diabetes affects males and females differently
- The diabetic rats were fed a basal diet. Another group could be fed with HFD
- Inflammatory and gut integrity markers were not analysed
- Faecal bacterial culture and 16S RNA profiling were not done. This could help in the profiling of gut microbiota.

CONCLUSION

LPS serves as a valuable biomarker for assessing gut dysbiosis in diabetes mellitus. The elevated levels of LPS in diabetic patients indicated increased gut permeability, allowing the translocation of LPS into the bloodstream. These toxins may play crucial roles in the worsening of glycaemic status. Thus, there is a need for further research to elucidate potential therapeutic interventions that could target gut permeability in the management of diabetes mellitus.

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Ethical approval: The study was approved by the Institutional Animal Ethical Committee (IAEC) of Sri Ramachandra Institute of Higher Education and Research Institute (SRIHER) (no: IAEC/69/SRIHER/823/2023, Date: 21st Februray 2023). The experiments were performed as per the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Declaration of patient consent: Patient consent is not required as there are no patients in this study.

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Conflicts of interest: There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation: The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript, and no images were manipulated using AI.

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