“Evaluate the effect following coadministration of Streptozotocin with High-Sucrose Fructose Diet on Fatty Acids Derivatives and Liver Pathophysiology”

A Dissertation Thesis Submitted to Institute of Science, Nirma University

In Partial fulfilment of the Requirement for

The Degree of

Master of Science In

Biochemistry & Biotechnology

Submitted by

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Under the Guidance of

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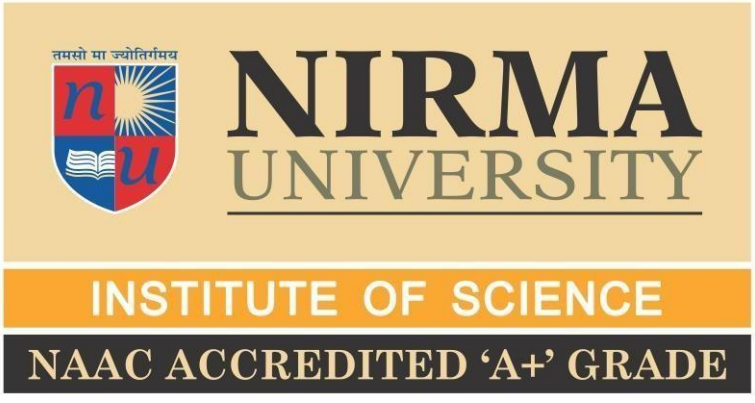

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CERTIFICATE

This is to certify that the thesis entitled "**Evaluate the effect following coadministration of Streptozotocin with High Sugar Fat Diet on Fatty Acids Derivatives and Liver Pathophysiology**" submitted to the Institute of Science, Nirma University in partial fulfillment of the requirement for the award of the degree of MSc. (Biochemistry/Biotechnology), is a record research work carried out by **Ms. Aradhna Vora (21MBC007), Ms. Malika Gehlot (21MBT018), Mr. Shivang Mehta (21MBT020)** under the guidance of **Dr. Sriram Seshadri**. No part of the thesis has been submitted for any other degree or diploma.

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Declaration

The above Dissertation project was carried out jointly by **Ms. Aradhna Vora (21MBC007), Ms. Malika Gehlot (21MBT018), Mr. Shivang Mehta (21MBT020)** under my guidance.

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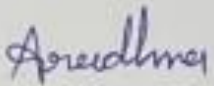
Date: 4th May 2023

Place: Ahmedabad

DECLARATION

We declare that the thesis "Evaluate the effect following coadministration of Streptozotocin with High Sucrose Fructose Diet on Fatty Acids Derivatives and Liver Pathophysiology" has been prepared by us under the guidance of Dr. Sriram Seshadri, Associate Professor of the Institute of Science, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.



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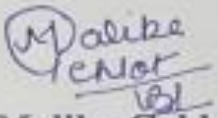

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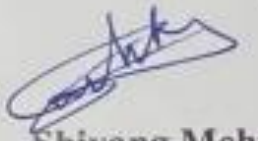

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"Presentation, inspiration, and motivation have always played a key role in the success venture."

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Finally, we would like to Thank God for letting us through all the difficulties.

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of variance
AUC	Area Under Curve
H&E	Haematoxylin and Eosin
HDL	High-Density lipoprotein
HPLC	High-performance liquid chromatography
HSFD	High Sugar Fat Diet
IR	Insulin Resistance
LDL	Low-Density Lipoprotein
LI	Large Intestine
LPS	Lipopolysaccharides
MCFA	Medium Chain Fatty Acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non- alcoholic steatohepatitis
NC	Normal Control
NF-kB	Nuclear Factor kappa B
OGTT	Oral glucose tolerance test
PB	Phosphate Buffer
PCR	Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TLR	Toll-like Receptors
Tm	Melting temperature

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ABSTRACT

ABSTRACT

Metabolic disorders are one of the -major concerned diseases worldwide. High sucrose fructose diet plays a major role in altering metabolism. When we talk about metabolic diseases like non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and Type 2 Diabetes. High sucrose fructose diet is one of the inducers of it. Reported data suggest, streptozotocin is being used to induce disorder like diabetes mainly type 1. In our study we wanted to analyse the morphological, biochemical and expression parameters in the animals fed with high sucrose fructose diet and high sucrose fructose diet along with different doses of streptozotocin for different duration of time period so the animals were divided in four different groups. Normal control, high sucrose fructose diet for 90 days, high sucrose fructose diet for 90 days along with mixed dose of streptozotocin and high sucrose fructose diet for 30 days and high dose of streptozotocin. And from our studies we found nearly significant results. The effect of high sucrose fructose diet and streptozotocin on the gut microbiota and their metabolites. As we know that in any metabolic disorder especially type 2 diabetes and inflammatory disease, gut microbiome alters. The population of gram-positive bacteria and gram-negative bacteria are concerned for the regulation of it or to prevent or induce disease condition. In-addition to this when we talk about the high sucrose fructose diet and gut microbiota, fatty acid derivatives also play a significant role. These fatty acid derivatives were also analysed using HPLC and the results came out to nearly relevant.

REVIEW OF LITERATURE

Liver:

Liver dysfunction is one of the major concerns these days, the most commonly occurring is a non-alcoholic fatty liver disease (NAFLD). Factors such as the deposition of fats in the hepatocytes, genetic predisposition, and HSFD intake are associated with liver dysfunction. Ingestion of a diet with excessive fat proportions may cause insulin resistance (IR), dyslipidemia, and metabolic and cardiovascular disorders. Research has suggested that the occurrence of NAFLD is a 2-hit mechanism. The first hit is mainly due to IR followed by the de novo lipogenesis and compromised transport of fatty acid. The second hit occurs because of stress, generated in the endoplasmic reticulum, mitochondrial dysfunctioning and apoptosis of hepatocytes which increases the chance of severe consequences such as non-alcoholic steatohepatitis (NASH), furthermore to cirrhosis, and may even lead to hepatocarcinoma. Studies have shown the relationship between NAFLD and other metabolic disorders, particularly with IR, type 2 diabetes mellitus (T2DM), and obesity (Abenavoli et al., 2016). Another common liver disease thriving is liver cancer or hepatic carcinoma. The reasons for the occurrence of hepatic carcinoma (HCC) are multifactorial. Investigations have shown that liver damage or injury in viral hepatitis does not occur directly due to the cytopathic effects of the virus but takes place due to the awakening of the immune response by the viral proteins. The immune system responds by the T-cell mediated mechanisms for direct disruption of the infected hepatocytes, by removing the viral particles from the circulation, by the antibody response, and lastly by the inflammatory responses. Recent studies show that activation of the inflammatory cytokines such as the NF-κB signaling is mainly responsible for liver injury and the development of tumors (Naugler & Karin, 2008).

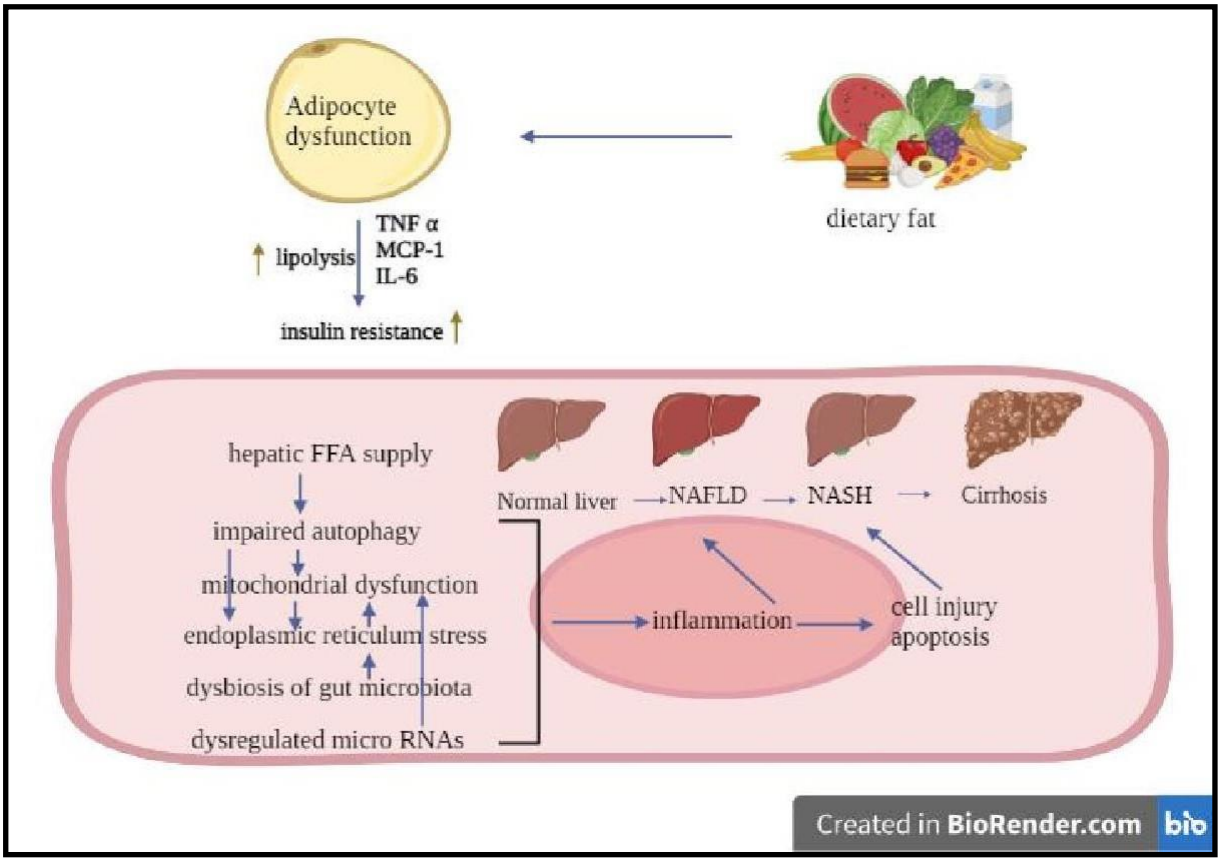

Figure 1. Liver damage due to high sugar high-fat diet.

High-sucrose fructose diet

High-sucrose fructose diets are mainly used for the induction of models for obesity and other metabolic disorders such as diabetes. These diets contain more amount of fats and are rich in calories. Such diets have high sacietogenic capacity so even though the food intake is reduced, then to a significant gain in weight and body fat is seen (White et al., 2013). These could vary in composition depending on the time and type of model to be established. The various types include the high-fat high-sugar diet, high-fat high sucrose diet, and high-fructose diet. Studies have shown that diet composition is very important as it is observed that, giving a diet with high sugar did not lead to changes that would characterize obesity whereas, a diet rich in fat, has shown that the adiposity index and the body fat increased when compared with the control or high sugar diet (Matias et al., 2018). Some studies have highlighted that the high-fat content were saturated fatty acids (Bortolin et al., 2018; Choi et al., 2016; Heo & Choung, 2018; Matias et al., 2018b; Picklo et al., 2017; Wu et al., 2018). Diets rich in saturated fatty acids and long-chain fatty acids can lead to a greater accumulation of fat and resynthesis of the TG (Lee et al., 2018). Such a type of diet can also trigger inflammatory cytokines which is generally the characteristic, observed in obesity (Cani, Amar, Iglesias, Poggi, Knauf, Bastelica, Neyrinck, Fava, Tuohy, Chabo, Waget, Delmée, et al., 2007; Lee et al., 2018). Reports suggest that due to HSFD certain hormones (cholecystokinine, peptide YY, and glucagon-like peptide-1) are triggered which reduces the diet intake (Ohlsson et al. 2014).

Diabetes:

Diabetes or Diabetes Mellitus (DM) is 3rd major cause of death worldwide. This occurs mainly due to an imbalance or unresponsiveness of the two hormones namely insulin and glucagon, which are necessary for maintaining the blood glucose level, produced, and secreted by the pancreas (Al-Hussein et al., 2021).

Type 1 Diabetes

Type 1 is an autoimmune disorder in which the beta cells of the pancreas responsible for the production and secretion of insulin are destructed by the body's own immune system. Due to this body fails to regulate blood glucose levels which leads to elevated glucose levels. This mainly reported in teens and children (Ma et al., 2019). The mechanism of liver damage in diabetes mellitus involves IR due to which lipolysis occurs in the peripheral adipocytes which cause the release of free fatty acids (FFAs) from the adipocytes into the circulation and their accumulation in the liver. Another factor contributing to the damage includes the release of pro-inflammatory molecules like the tumour necrosis factor-α (TNF-α) and leptin from the adipocytokines which increases the damage to the hepatocytes, due to the occurrence of oxidative stress in the mitochondria (Manna et al., 2010a; Palsamy et al., 2010). This connectional action of the mitochondrial oxidative stress, hyperinsulinemia, and hyperglycemia leads to the production of free radicals that causes inflammation and cellular necrosis. This inflammation triggers the production of collagen in the hepatocytes (stellate cells), worsening the condition causing fibrosis, cirrhosis and eventually leading to hepatocellular carcinoma.

Type-2 Diabetes

Type-2 diabetes is a commonly observed diabetes, around 90-95% of people fall under this category of metabolic disorders in adults nowadays. The condition occurs in the body when the cells cannot uptake glucose due to insufficient binding activity between insulin and its receptors present on the cell surface. The IR in the liver results in hyperglycemia causing problems in glucose metabolism(SHASHI & PK, 2022). The liver fails to detect the available glucose despite extreme hyperglycemic conditions and produces more via activity of certain enzymes such as glucose-6-phosphate dehydrogenase, fructose-1,6-di phosphate, hexokinase and glucokinase. Glucose homeostasis is also regulated by the expression of certain genes which encode for hepatokines. The hepatokines namely fetuin-A, betatrophin/angiopoietin-like protein 8 (ANGPTL8) and fibroblast growth factor 21(FGF21)(Iroz et al., 2015; Stefan et al., 2014). Studies has shown that these hepatokines further exacerbate diabetic progression. Another researched aspect shows that the liver stops oxidizing fatty acids and use them to synthesize TG which then starts to accumulate in the liver causing damage.

Streptozotocin:

Streptozotocin (STZ) is a chemical produced by a microbe called *Streptomyces achromogens* present in the soil. It also acts as an antibiotic for gram-negative bacteria. Its toxicity towards the beta cells of the pancreas was reported in the mid-1970s and this increased its use in the research field in order to establish the model includes being used as a chemotherapeutic agent in treating pancreatic cancers and is known as zanosar. STZ is a white-colored powdered, odorless chemical which completely soluble in water and partially soluble in polar solvents. The half-life of this chemical has been reported to be 15 minutes and it is light sensitive. The IUPAC name of this compound is 2-deoxy-2-[[(methylnitrosoamino)carbonyl] amino]-d-glucose. This damages the genetic material by transferring its N- nitroso group to the cells of the pancreas, thereby disturbing their DNA synthesis mechanism. Many reports suggest that rather than damaging the pancreatic cells, STZ affects the nucleotide synthesis and certain enzymes which are important for glyconeogenesis and leads to the activation of the poly ADP-ribosylation which is the main cause of induction for diabetes. As STZ is known to facilitate glucose transport in the cell with the help of glucose transporter-2 (GLUT 2). The abundance of this transporter on the beta cells of the pancreas is one of the main reasons that is responsible for this destruction that leads to diabetes (llahi & Hosseini, 2014).

Gut Microflora:

Gut microbiota is the population of microorganisms thriving in one environment. These microbes harbour a thousand times more genes than the human genome. These genes encode a lot number of proteins that are not translated within the human genome. These proteins play a crucial role in physiological aspects that is vital in establishing balanced metabolism (Hooper and Gordon, 2001). With the current advancement in technology, deep dig in research has explored the genera of the microbes residing within the gastrointestinal tract. The sequencing techniques employed include the 16S ribosomal (rRNA), whole genome shotgun, and metagenomic assembly. Gut microbiota includes manifold functionalities. Among them, one most important functions of the gut microbiota involve the alteration of energy homeostasis as well as the development of free fatty mass. The interaction between the gut microbiota and the host is quite complex where there is a large no. of crosstalk that takes place including the immune system along with the neuroendocrine system. This interplay among these is generally mediated by the metabolites that are produced by the microorganisms. Despite the many advantages of the microbes these many a times come out to be responsible for pathological conditions. Research has reported that in dysbiotic condition certain set of the microbiota involved in metabolic disorders namely diabetes, obesity, and NAFLD. Previously the gut microbiota has been thought to be static in T2DM an metabolic disorder but with the advancement in research, it is revealed that they play an important role in many diseases and disorders. The gut microbes and their interaction with the host have brought them to the sphere of attention. Alteration of the gut microbiota is one of the main factors for characterization of the metabolic disorders mainly obesity and the T2DM along with low grade inflammation, changes in the gut permeability and barrier which may cause metabolic endotoxemia i.e., the release of the endotoxins such as the LPS in the bloodstream, that triggers inflammation (Everard et al., 2013). Consumption of diet with excessive fat can lead to changes in the composition of the gut microbiota (Bortolin et al., 2018)

Higher population of gram-negative bacteria induces inflammation, gut permeability, insulin resistance, metabolic endotoxemia, histological impairment of tissue such as adipose, gut barrier, and gut peptide secretion.

Research has shown that category of fatty acid namely SCFA known for pivotal. These are organic acids comprising 1-6 carbon atoms aligned in straight or branched chain conformations (Miller & Wolin, 1979). These are produced in the gut by the fermentation of dietary fibres by

the gut microbiota. Commonly reported SCFA are acetate, propionate, and butyrate (Topping & Clifton, 2001). Many studies have shown that the ratio of these fatty acids depends upon the type of diet intake. A diet rich in fiber causes an increase in the production of SCFA whereby the HSFD causes reduced production of these fatty acids (Bergman, 1990; Topping & Clifton, 2001). SCFA has also been reported to provide protection against the diet-induced insulin resistance and obesity (Lin et al., 2012) .

A high-sucrose fructose diet may be one of the reasons for NAFLD which occurs due to the excessive accumulation of fats in the liver. Which most prevalent condition (Katsagoni et al., 2018). The fats from the diets get stored in the adipose tissue. The adipocytes are fat storing cell that store fat a certain extent, deposition of fats beyond the capacity of these cells releases fats, from these cells into circulation which result in inflammation, insulin resistance, and other metabolic disorders such as obesity and T2DM. It is also been reported that a HSFD causes the alteration of the gut microbiota and causes inflammation. These alterations in the gut microbiota include a decrease in the population of *Bacteroidetes* and an increase in the population of *Firmicutes*. It has also been reported that the animal model fed with HSFD for a span of 60 days shows metabolic syndromes linked to oxidative conditions (Cani, Amar, Iglesias, Poggi, Knauf, Bastelica, Neyrinck, Fava, Tuohy, Chabo, Waget, Delmée, et al., 2007).

The study also reveals that the insulin resistance which occurs due to the accumulation of the fats may destroy the hepatocytes and the hyperglycaemic condition can cause the disruption of the lipid metabolism can stimulate the cascade of inflammation (Auberval et al., 2014).

A low dose of STZ through injection along with the HSFD has been reported to cause the destruction of the beta cell of the pancreas and reduce its functional capacity (Gheibi et al., 2017).

When STZ is metabolized carbonium ions are generated which damages the DNA by alkylation or by forming inter-strand crosslinks in the DNA (Wilson & Leiter, 1990; Weiss R. B. 1982).

The increased ROS leads to a loss of the balance between the pro-oxidant and antioxidant defense strategies of the body and causes the body to be in the condition of oxidative stress (Eleazu et al., 2013).

Studies suggest that STZ-induced diabetes mainly occurs through the formation of reactive oxygen and nitrogen species. And these reactive species are known to cause oxidative stress in the body (Nukatsuka et al., 1988; Raza et al., 2011).

It has also been reported that STZ dose leads to a Change in the morphology of the liver and its enzymes (Manna et al., 2010b). The investigation has showed that apart from the toxicity of the vital organs a dose of STZ of 65mg/kg has caused gastro-mucosal ulcerations. Further, it is also shown that higher doses of STZ cause lower muscle mass as well as bone volume an increase in marrow adiposity is seen (Bleasel & Yong, 1982). Lower dose of STZ linked in lowering of total body weight while an increase in the weight of organs, such as the liver and kidney was seen but no change in the weight of the pancreas. Observation of cytotoxicity in the hepatocytes due to oxidative stress and mitochondrial dysfunction has also been made. (Turk et al., 1993).

HYPOTHESIS

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HSFD is known to cause gut dysbiosis which leads to inflammation. HSFD in combination with Streptozotocin is also being used for model induction to study Type-2 diabetes.

Here in our study, we hypothesize that the HSFD and STZ used for model induction may cause alteration in gut microflora, and their diversity and gut derive metabolites such as fatty acid derivatives. What is the key player linked with T2D and associated complications, in the form of Biochemical changes, tissue morphology, and gene expression (Figure 2).

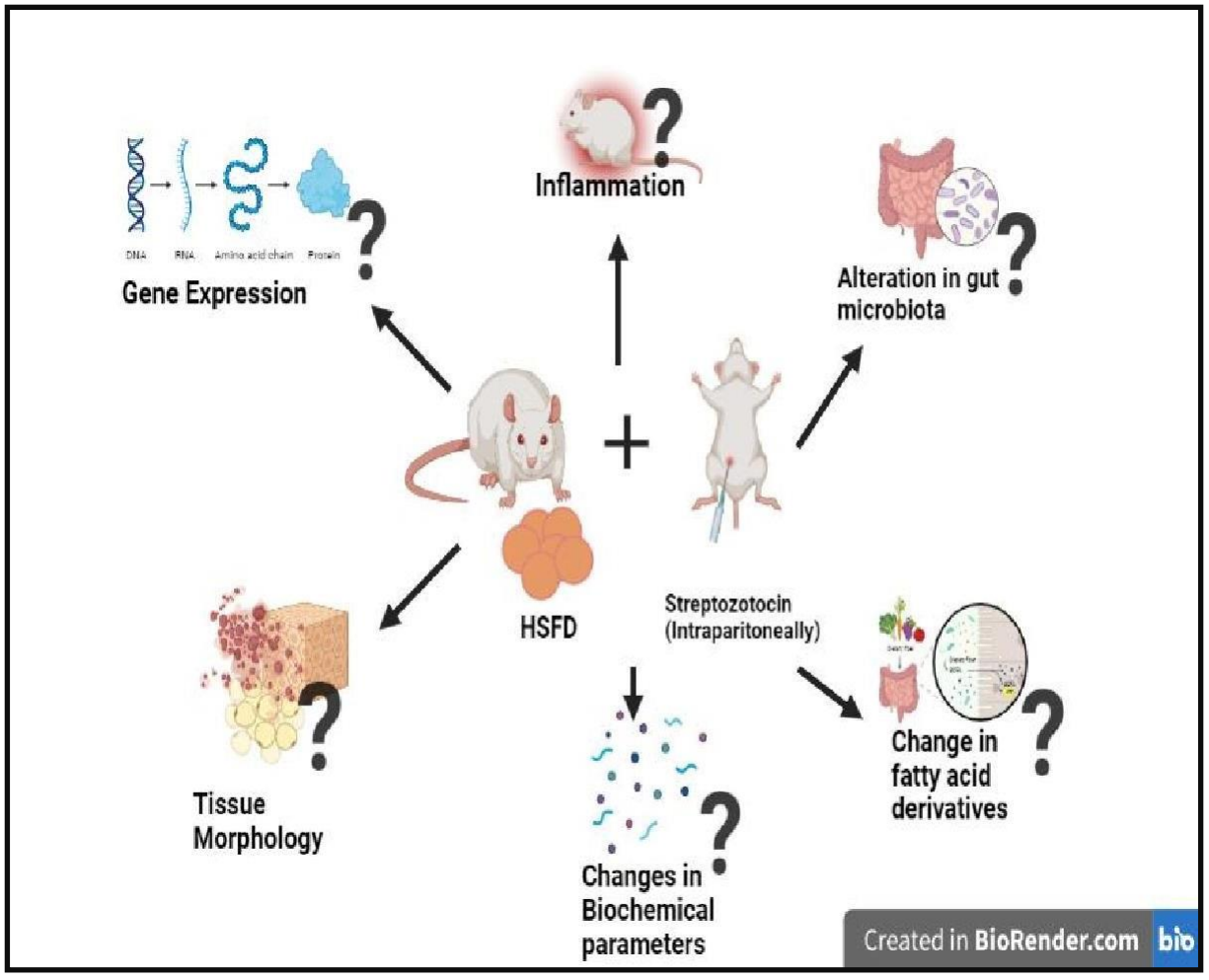

Figure 2. Illustration of Hypothesis

OBJECTIVES

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1. To evaluate and compare the biochemical, morphological, and expression parameters in animals fed with high sucrose fructose diet with and without Streptozotocin.
2. To evaluate the fatty acid derivatives and correlate them with the gut microbiota in the study groups.

MATERIALS AND METHODS

MATERIALS AND METHOD:

Animals and ethical consent:

For the study, the male Wistar rats were procured from Zydus Research Center, Ahmedabad. The average weight of the animals was 300-450 g. These were housed in the central animal facility situated in the Institute of Pharmacy Nirma University. Prior to the study the animals were quarantined for 2 weeks. The rats were kept in cages and were fed with chow pellets and RO drinking water. The temperature and the humidity of the central animal facility were properly maintained. The study was carried out in accordance with the ethical guidelines for the care and use of laboratory animals of institutional Animal Care and Use committee Nirma University, Ahmedabad under the CPCSEA guidelines of the Ministry of Environment and Forest New Delhi. Protocol No. (IS/Ph.D.30/2022/31). The animal grouping is as follows (Table1).

Table-1 Animal Grouping

Group Name	No. of animals
Normal control	3
HSFD induced diabetic group	3
HSFD + 40mg streptozotocin	3
HSFD + 50mg streptozotocin	3

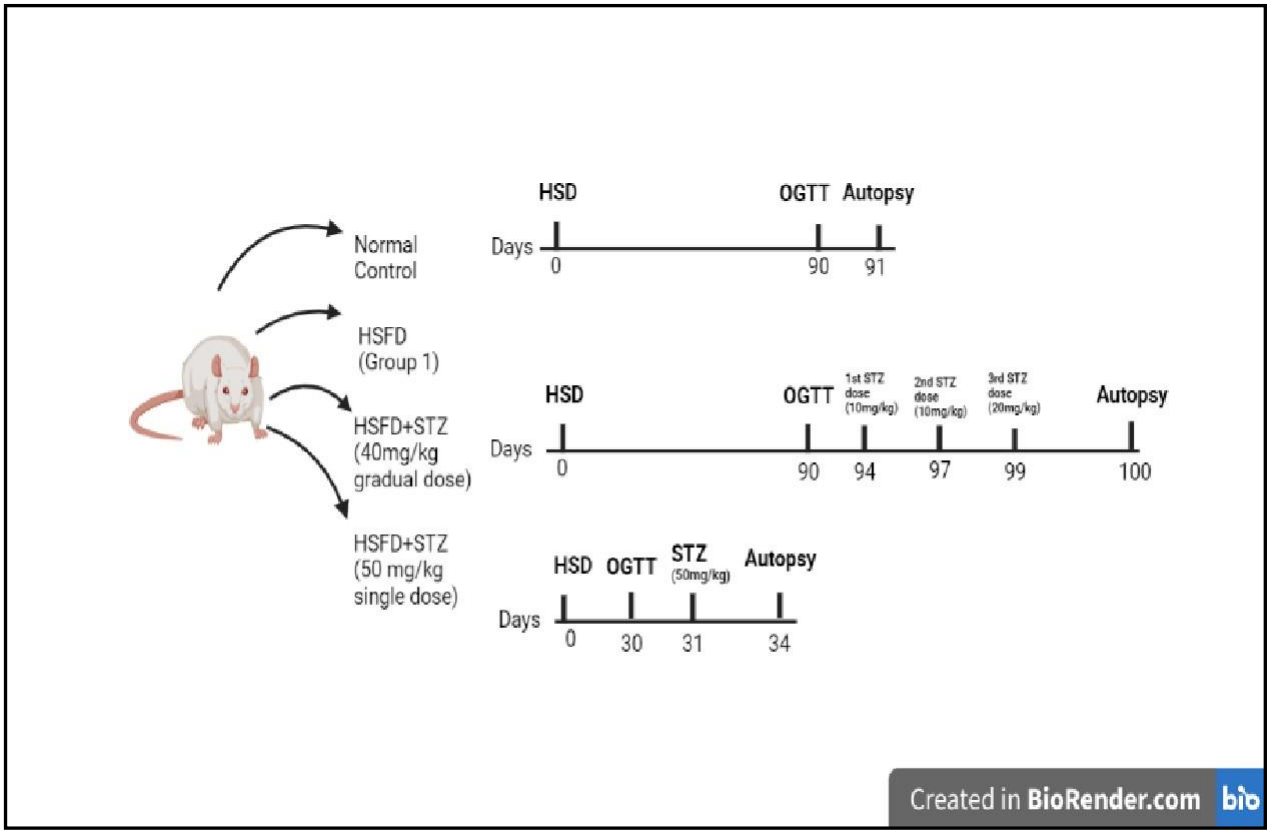


Figure 3. Animal grouping and timeline of study.

Table-2 Composition of HSFD

Composition For 500 gm	
Wheat bran	25 gm
Ground nut oil	25 ml
Casein	100 gm
Methionine	1.5 gm
Salt mix	17.5 gm
Vitamin mix	5 gm
Choline chloride	1 gm
Dalda	150 gm
Sucrose	75 gm
Fructose	87.5 gm

The diet was prepared by mixing the aforementioned ingredients in the amounts mentioned in Table 2.

Oral dosing

65% sucrose solution was prepared in distilled water and was given orally using a gavage tube twice daily.

Fructose Dosing

4% Fructose water was prepared in distilled water by weighing 8g of fructose and adding it to 200 ml of distilled water.

OGTT:

OGTT is used to quantify how effectively the body can process an immense amount of sugar. If the blood sugar measured in the test is above a definite level, this could be a sign that sugar is not being metabolized enough by the

body's cells. First, the animals were kept starved for twelve hours. After 12 hours the first reading was taken which was said to be the reading at 0 min. After that dextrose solution 2x the body weight was given orally using a cannula, then the readings were taken at 30 min, 60 min, and 120 min. Further graphs were plotted and the results were analyzed.

Fecal collection:

Collection of the fecal was done periodically and the samples were stored at -80ºC until use. Further these samples were used for isolation of the bacterial DNA and to obtain the information of the bacterial population present in the samples (SCFA).

Streptozotocin Dosing:

Streptozotocin is widely used to induce diabetic models (usually type-1). STZ doses were administered to the animal intraperitoneally. The STZ was dissolved in citrate buffer. The PH of the buffer solution was maintained at 4.5-5. The dosage was calculated as per the weight of individual animal. The mixed dose was administered thrice a week according to BW of animal and the dose is 10mg/kg, 10 mg/kg, and 20mg/kg. The first and second doses administered were of 10mg/kg followed by 20mg/kg to the animals after 90 days of induction. To another group, a single dose of 50mg/kg STZ was administered in rats one after one month of HSFD induction.

Autopsy Schedule:

We have scheduled the autopsy on basis of the completion of our induction time-line. The procedure set based on the data from review of papers for the model induction. The animals were euthanized according to the groups in which they were divided. The group in which high sugar fat diet is given for 90 days were sacrificed on 91st day. Animals from the high sugar fat diet (90days) with mixed dose of Streptozotocin (10mg/kg, 10mg/kg, 20mg/kg) were sacrificed on the 100th day as mix-dose is given till the 99th day with observation in-between. Animals from the group in which high sugar fat diet is given for 30 days and a single shot of streptozotocin(50mg/kg) was administered in model on 31st day and after two days of observation the animals were euthanized.

Serum separation:

Blood was drawn from the euthanized animals by puncturing the heart and liver using a syringe. The blood was centrifuged on high speed and extracted serum was used for, biochemical analysis including TG (Triglycerides), SGOT (Serum Glutamate Oxaloacetate Transaminase), SGPT (Serum Glutamate Pyruvate Transaminase), and Glucose, using the manufacturer's protocol for the Lab-care diagnostics kit that we used for the experiment.

DNA isolation from fecal sample:

DNA was isolated from the collected fecal sample using QIAamp® Fast DNA or Stool Mini Kit (cat no. 51604)

High-performance liquid chromatography (HPLC):

For fatty acid derivatives analysis, HPLC was performed. The samples were prepared using fecal samples. These fecal samples were vortexed in the PB buffer of 2.5 pH and were then centrifuged at 5000 rpm for 15 minutes. This homogenate was then filtered using the syringe filter. Approximately 10 µl of the sample was injected for the analysis. Standards for SCFA (acetate, butyrate, and propionate) and MCFA (hexanoic and octanoic acids) were also prepared using the PB buffer. Keeping the concentration of 100 mg/ml. The run time for each sample was kept 15 minutes.

Histopathological Analysis:

During the autopsy, a small section of the large intestine, liver, and adipose tissues from the dissected animals were collected and the tissue was fixed using 10% formaldehyde, and were given for H&E staining. The remaining tissues were stored at -20ºC deep freezer for the longer storage and further analysis.

Gene expression and analysis studies:

RNA isolation:

RNA was isolated from liver tissue, which was then quantified using the nanodrop spectrophotometer and was then used for the cDNA synthesis.

For RNA isolation, tissues stored at -20º C and approximately 100mg of the tissue was taken in the homogenizer tube to which 1000 µl of RNA iso plus was added and the homogenization was done. The homogenate was then transferred into 2 ml eppendrof and was kept on ice for 5 minutes. After this, the eppendrof was centrifuged at 12000 rpm for 5 minutes at 4ºC. The supernatant was then transferred carefully to another 1.5 ml eppendrof and 200 µl chilled chloroform was added, this was then mixed gently and was kept at room temperature for 5 minutes (the milky suspension was formed). The eppendrof were then again centrifuged at 12000 rpm for 15 minutes at 4ºC. The supernatant (100-200 µl) was then collected into fresh 1.5ml eppendrof and then to this 200 µl chilled isopropyl alcohol was added and was again centrifuged at 12000 rpm for 10 minutes at 4ºC. The supernatant was discarded carefully and the pellet obtained was washed with 200 µl of ethanol. The eppendrof were then centrifuged at 8000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet obtained was air dried in the ice box for 10 minutes. The pellet was then dissolved in 75 µl of nuclease free

water and was loaded on the gel. The ratio and the concentration of the RNA was then measured using the nanodrop and further processing was done for the cDNA synthesis and the gene expression studies.

Gene	5'-3'sequence	Temperature °C
TLR-4	F: TGCAGAGCAACGATGGAGAAA R: ACAGCAGCGTCAGGGTGAAG	F: 68.1°C R: 67.9°C
β-actin	F: AGTGCTGTGGGTGTAGGTAC R: GCAAAGAGGGCAAGAACACA	F:59.03 °C R:58.97 °C
rpoD	F: CGTGTGCCTCTTCTTACTGC R: TCTACGGCCTTCATCAGACC	F:58.93°C R:58.93 °C

RNA for the expression of TLR 4 was checked by running real time polymerase chain reaction (RT-PCR), for this the cDNA synthesis was done using the Thermo fisher First strand cDNA synthesis kit.

Primer Designing for gene expression studies

The primers for the desired genes were designed using online platforms. The Fasta sequence of the desired genes was obtained from NCBI. These sequences were then used for designing primers using the online tool Primer 3. The primers fulfilling all the required properties were selected and were further analyzed in another online tool oligocalc and primer stat to check for any self-complementarity or hairpin loop structures. Then the UCSC in silico PCR was used to check the amplicon length.

Table – 3 Primers for gene expression studies

Microbial quantification by RT-PCR

The microbial DNA was isolated from the fecal samples and gel electrophoresis was done to check for required DNA bands. After this the DNA samples were quantified using nanodrop, and the PCR was done using primers specific to 16s rRNA genes.

Table-4 Primers for microbial quantification.

Name	5'-3'sequence	Temperature °C
Lactobacilli	F- GCGTGCTTAACACATGCAAGTC	65°C
	R- CACCCGTTTCCAGGAGCTATT	63.2°C
Bifidobacteria	F-CATGCCGCGTGTATGAA	66.7°C
	R-CGGGTAACGTCAATGAGC	66.1°C

Forward and reverse primers sequence of bacteria were used in the study with standardized Tm.

Statistical Analysis:

The analysis of all the data was done using one way analysis of variance. The data was compared multiple times using Turkey's test. All values are expressed as mean ± SD. * and $^{\#}$ depicts significance level, * or $^{\#}$ $p<0.05$, ** or $^{\#\#}$ $p<0.01$ and *** or $^{\#\#\#}$ $p<0.001$. All the data was analyzed using Graph Pad Prism (version 5.0.1).

RESULTS

Physiological Parameters:

Body weight

There were no significant changes observed in the body weight and liver weight within the groups. However, it was observed that the normal control group gained some amount of body weight as compared with the groups fed with HSFD, HSFD-90d-STZ 40mg/kg and HSFD-30D-STZ 50mg/kg (Figure 4 A). Weight of liver can be seen higher in HSFD-90D-40mg/kg compared to HSFD and HSFD-30D-50mg/kg.

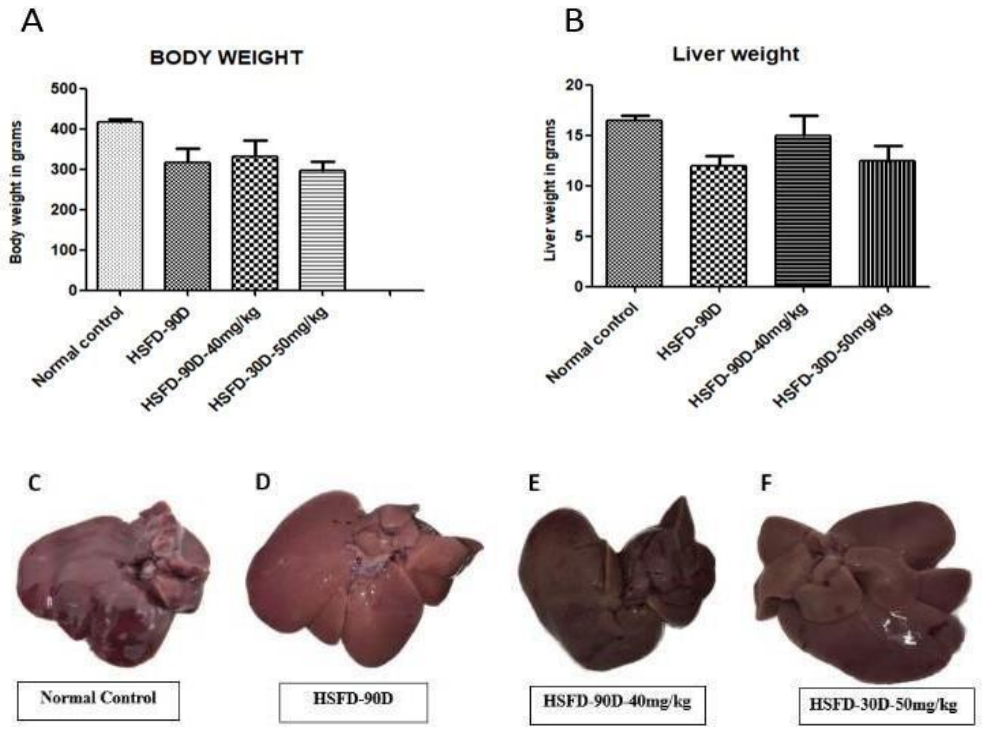


Figure 4: Physiological parameters of group NC, HSFD-90D, HSFD-90D-40mg/kg, HSFD-30D-50mg/kg where, **(A)** Body Weight **(B)** Liver Weight. Data are presented as Mean ± SD, N for each group. One-way ANOVA was performed for liver weight. GraphPad Prism (v5.01) was used for statistical analysis. (**C-F)** gross imaging of liver.

Serum biochemical profiling

Serum biochemical analysis of glucose, (TG) Triglycerides, HDL (High-Density Lipoprotein), LDL (Low-Density Lipoprotein), SGOT (Serum Glutamate Oxaloacetate Transaminase), and SGPT (Serum Glutamate Pyruvate Transaminase) tests were performed, with the help of protocol as provided by the manufacturer of the diagnostic kit (Accucare reagent kit by Lab-care diagnostics).

Table-5 Serum profiling for NC, HSFD-90D, HSFD-90D-40mg/kg, HSFD-30D-50mg/kg group.

Serum Biochemical	NC	HSFD-90D	HSFD-90D-40 mg/kg	HSFD-30D-50 mg/kg
Glucose mmol/L	44.13 ± 1.42	7.35 ± 3.44**	28.15 ± 8.32$^{\#}$	32.86 ± 11.67$^{\#}$
TG (mg/dl)	72.46 ± 2.17	150.94± 22.04	136.56 ± 48.95	214.83 ± 77.87**
SGPT (mg/dl)	51.76 ± 34.20	37.3± 4.03	21.77 ± 9.63	32.86 ± 15.28
SGOT (mg/dl)	90.92 ± 52.41	77.07 ± 30.50	123.87 ± 44.175	129.81 ± 65.38
Cholesterol (mg/dl)	70.065 ± 3.48	103.71± 30.28	128.39 ± 38.78	80.51 ± 64.16
LDL (mg/dl)	76.67 ± 4.70	35.52 ± 7.73**	60.98 ± 12.61	41.20 ± 13.86**
HDL (mg/dl)	46.42 ± 8.65	71.42 ± 21.94	37.58 ± 13.85	57.98 ± 11.37

The above table depicts the results of the serum biochemical analysis. The data are represented as mean ± SD. The data were analysed using one-way ANOVA where ** represents a comparison with the normal control group and significance is presented as **$p<0.01$. The $^{\#\#}$ represents comparison between HSFD-90D-40mg/kg and HSFD-30D-50mg/kg.

As shown in table no 5, LDL decreased in HSFD and HSFD-30D-50mg/kg than the normal group. As compared to HSFD groups, the levels of glucose were low in HSFD-90D-40mg/kg and HSFD-30D-50mg/kg.TG level inHSFD-30D-50mg/kg showed significant spike when compared to NC. Cholesterol levels were higher in the group HSFD-90D-40mg/kg in compared to other three groups but data is not significant. Other

biochemical parameters did not show any significant data when compared to NC or HSFD. A greater number of animals would be required further to generate more reliable data.

OGTT:

The oral glucose tolerance test was performed and the utilization of the glucose was checked after the oral dosing of dextrose (2g/kg) to the animals fasted overnight. The levels of glucose were checked at time intervals which were 0, 30, 60, and 120 minutes. Firstly, the rise in the glucose levels in the 30 minutes was observed in the normal control animals which then became normal at 120 minutes. The rise in the glucose level was observed in all animals but after the time duration of 120 minutes, the glucose levels were normalized in all the groups.

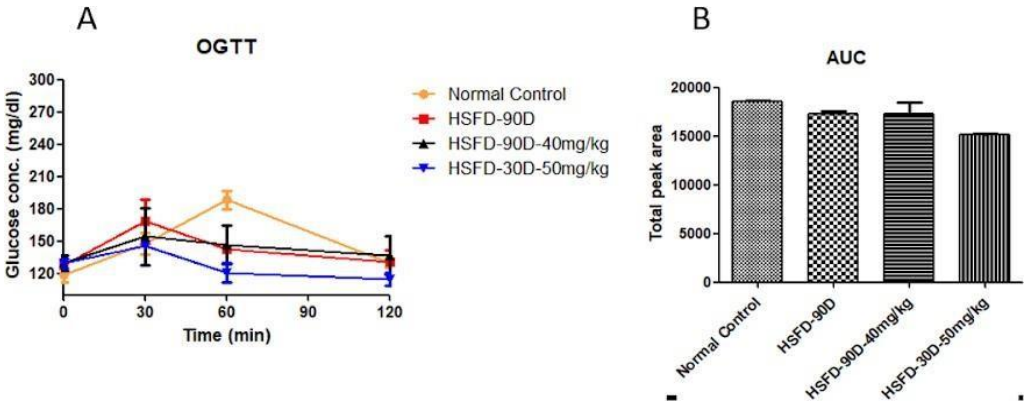


Figure 5. A, shows the OGTT results of the groups namely NC, HSFD-90D and HSFD-90D-40mg/kg and HSFD-30D-50mg/kg. Figure 5 B is statistically analyzed AUC.

Analysis of Fatty acid derivatives:

From the obtained result as shown in the figure 6. (A to E), we can interpret that the levels of both SCFA and MCFA are higher in the samples in comparison with the normal control. The concentration of SCFA should be lower in the disease condition according to the reported data. Although the level of propionate can be seen higher in normal control. Caproic acid shows the significant result when HSFD-90D-40mg/kg is compared with NC, the significance is indicated by (*) ($p<0.05$) in the figure 6(D). Change in this Fatty acid is also compared with the HSFD-90D and HSFD-90D40mg/kg and HSFD-30D-50mg/kg and it is denoted by the sign # in the figure 6 (E). Caprylic acid shows the most prominent values when group HSFD-30D-50mg/kg is compared with the normal control, and it is denoted as ***. The significance is represented as *$p< 0.05$, ***$p<0.001$.

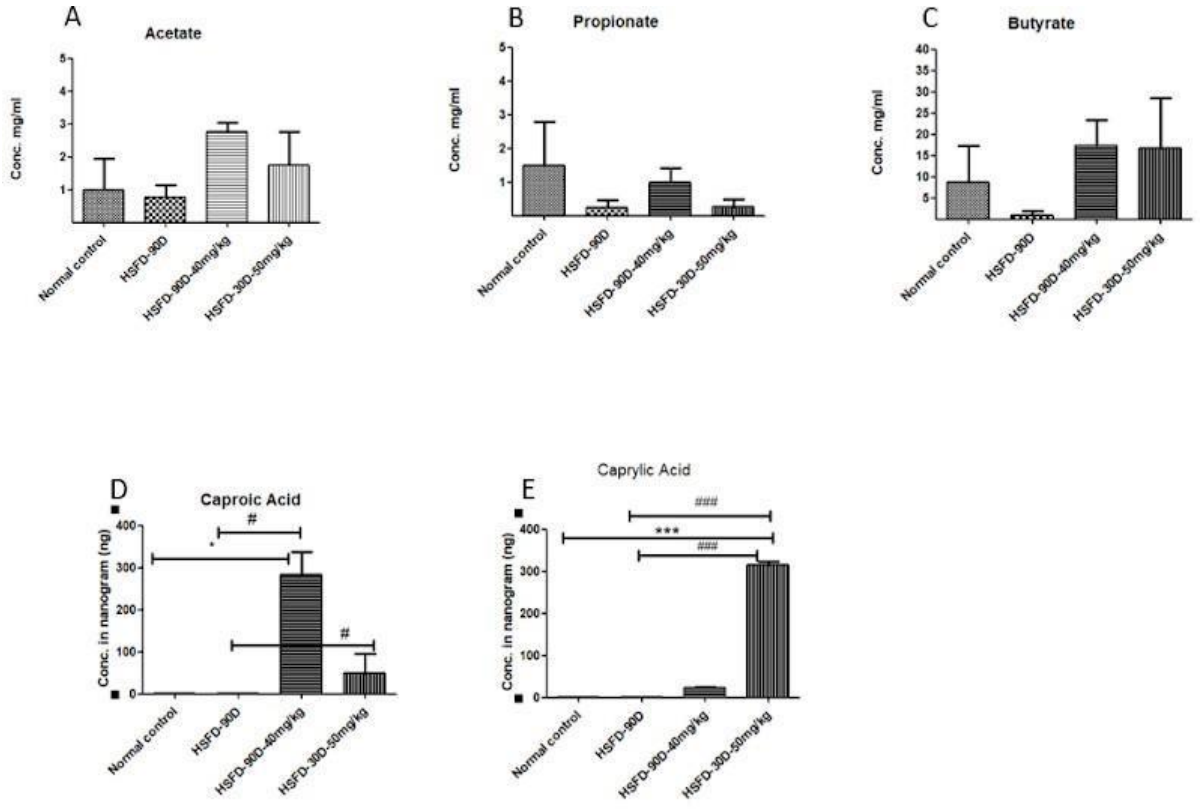


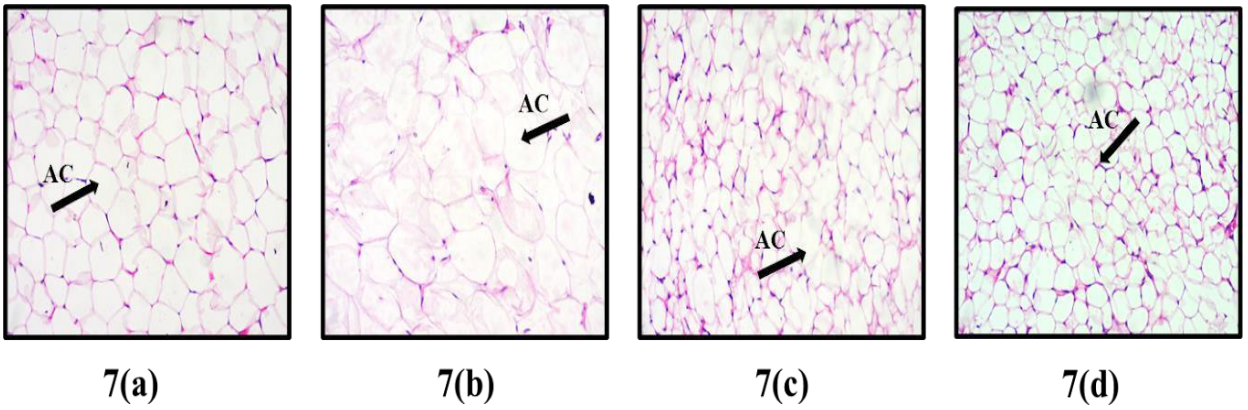
Figure 6. is the representation of the concentration of fatty acid derivatives present in the group NC, HSFD-90D, HSFD-90D-40mg/kg, HSFD-30D-50mg/kg. Figure 6 A to C shows the acetate, propionate, and butyrate respectively while D and E represents caproic acid and caprylic acid. Significance in graph D and E is defined as, *$p<0.05$, ***$p<0.001$ for comparison

of HSFD-90D-40mg/kg with NC and $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ for comparison of HSFD-90D-40mg/kg and HSFD-30D-50mg/kg with HSFD-90D.

Histology analysis:

The figurers 7 to 9 illustrate the images of Adipose, Liver and Large intestine respectively. The indication (a) to (d) depicts group normal control, HSFD-90D, HSFD-90D-40mg/kg, HSFD-30D-50mg/kg. The HSFD-30D-50mg/kg shows the inflammation and disruption in all the three tissues at greater note in compare to other groups as it can be seen from the figures.

1. Adipose:



7(a) 7(b) 7(c) 7(d)

Figure: 7(a) NC; compact and uniform size of AC seen, 7(b) HSFD; less compact and size variation in adipocyte, 7(c) HSFD+40mg/kg STZ; loosely compact and distorted AC seen, 7(d) HSFD+50mg/kg STZ; variation in AC size and loosely contact with neighbour cell. AC: Adipocytes**.**

2. Liver:

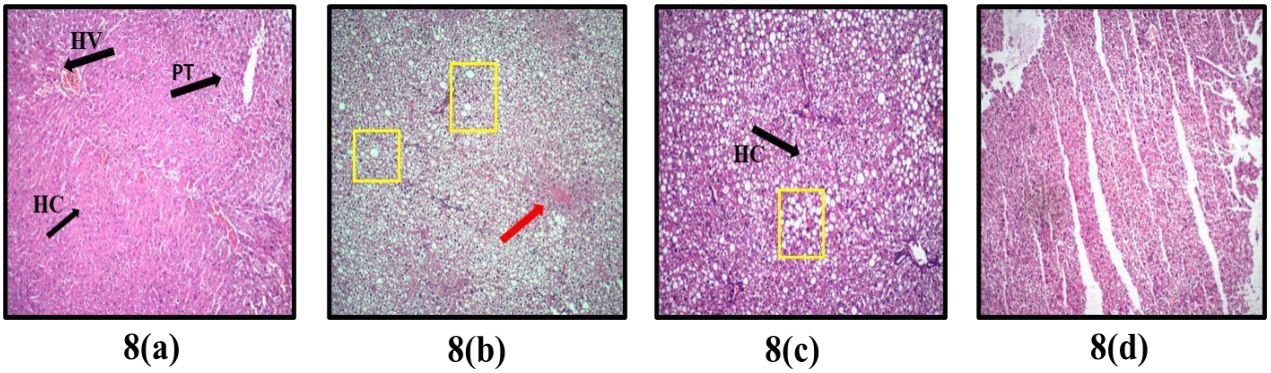


Figure: 8(a) NC; normal HC seen, 8(b) HSFD; fat globules and inflammation seen, 8(c) HSFD+40mg/kg STZ; larger fat globules seen, 8(d) HSFD+50 mg/kg STZ; distorted HC seen. HV: hepatic venule, HC: hepatocytes, PT: portal tract. Red arrow shows inflammation and yellow rectangle shows fat globules present liver.

3. Large intestine:

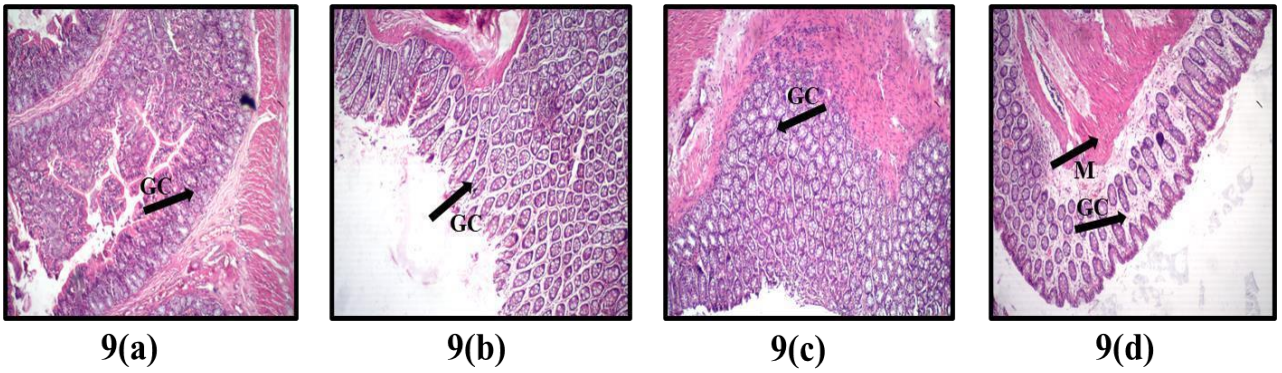


Figure:9(a) NC; compact and uniform size GC, 9(b) HSFD; loosely compact of GC, 9(c) HSFD+40mg/kg STZ; smaller size of GC seen, 9(d) HSFD+50mg/kg STZ; disrupted GC seen. GC: globular cell, M: mucus.

Gene Expression Analysis:

The expression for the TLR4 was examined in the groups NC, HSFD-90D, HSFD-90D-40mg/kg. The sample from group HSFD-30D-50mg/kg was not examined. The results obtained shows an increase in the relative abundance mRNA expression in the group HSFD-90D-40mg/kg (Figure 10). In order to analyse in which group expression of TLR4 is upregulated further study is required.

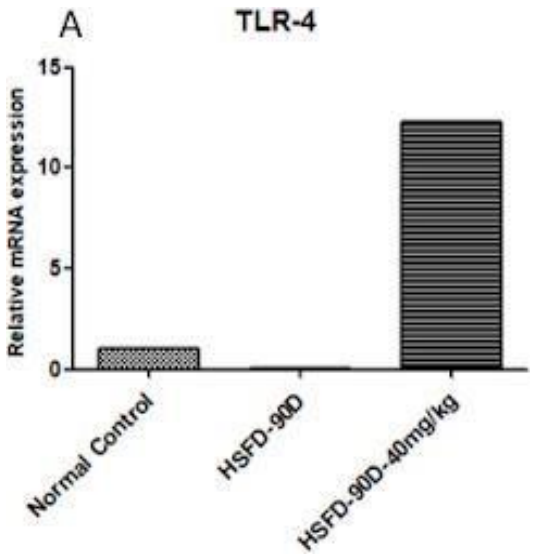


Figure 10. Relative mRNA expression of NC, HSFD-90D, HSFD-90D-40mg/kg.

Microflora Analysis

The results obtained shows the relative abundance of the *Bifidobacterium* in both the group is less in comparison where as an increase in the abundance could be seen in the *Lactobacillus* in the group given the mixed dose of STZ.

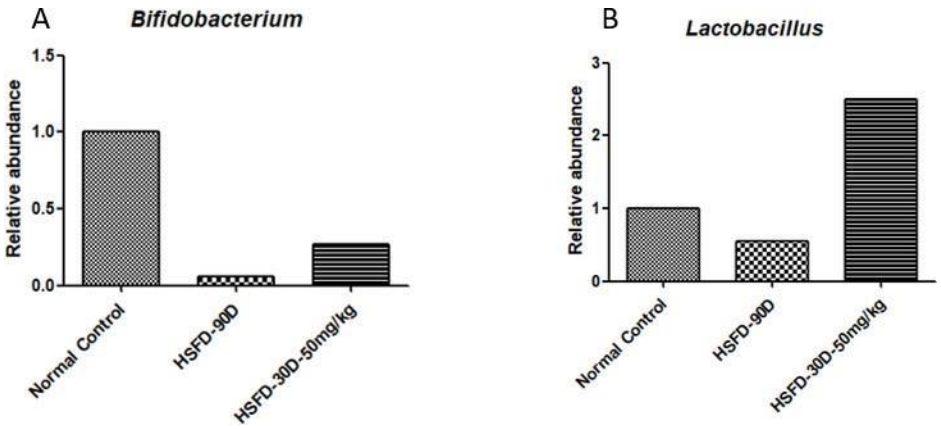


Figure 11: Relative Abundance of microflora in group NC, HSFD-90D, HSFD-30D-50mg/kg where figure A represents *Bifidobacterium* and figure B represents *Lactobacillus.*

DISCUSSION

Discussion:

Animals exhibited insulin resistance when dietary fat caused adipocyte malfunction, which led to lipolysis. Impaired autophagy occurs in the liver as a result of the supply of free fatty acids brought on by HSFD. Dysregulation of ER stress, gut dysbiosis, and microbial dysfunction all contribute to inflammation, which in turn causes cell damage and apoptosis (White et al., 2013). In our studies we observed that low dose STZ along with longer exposure to HSFD showed fat accumulation and high dose of STZ along with HSFD for short duration showed disruption of hepatocytes. This is clearly been explored in the histological examination that HSFD has caused expansion and disruption of the adipocytes but on the other hand the groups given, different dosage of STZ with different duration of HSFD, showed no significant change when compared to NC. In the case of liver, the histological imaging represents fat accumulation in the hepatocytes in the HSFD-90D group. Where as in the HSFD-90D-40mg/kg, clear inflammation along with accumulated fat in the hepatocytes can be seen and the group HSFD-30D-50mg/kg shows destruction in the same.

The levels of glucose and LDL were low in our induced models, when compared with NC. Whileno impairment in fasting as well as random blood glucose levels were observed, as the OGTT and the serum glucose levels did not demonstrate any sort of relevance. Studies suggested that TG levels should be high owing to HSFD and STZ dosage (Wang et al., 2011). HSFD with 50 mg/kg STZ demonstrates the resemblance to the reported data. We may infer from the data at hand that HSFD with a high dose of STZ can raise levels of TG in blood, but animal studies with a greater number of animals are required for more substantial results.

Investigation has shown that during dysbiosis the gut microbiota gets altered due to which the levels of the SCFA goes down (Bergman, 1990; Topping & Clifton, 2001). From the fecal sample analysis, we can say that the resultant proportions of acetate and butyrate were high but low propionate levels were observed. Due to this insignificance, further validation by the continuous study is required. While considering, it was seen that the amount of caproic acid was high in HSFD with 40 mg/kg STZ group and on the other hand caprylic acid has shown rise in group HSFD with 50 mg/kg STZ group. Further the microflora

CONCLUSION

Conclusion

In our studies we observed that the liver was highly affected by the HSFD along with STZ. The duration of the diet and the dose of STZ played an important role, as the groups HSFD-90D, and HSFD-90D-40mg/kg showed fat accumulation in the hepatocytes whereas in HSFD-30D-50mg/kg group the liver damage has been observed.

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APPENDIX-I

High Performance Liquid Chromatography:

For the HPLC the standards were made in filtered phosphate buffer(1X). The standards were weighed 100mg/ml for acetate, butyrate, and propionate. Hexanoic acid and octanoic acid were measured 60µl in 600µl as both the MCFA are in liquid form. All the standards were ordered from Sigma Aldrich. The samples were taken in concentration of 150mg/1.5ml in run for SCFA and 200mg/2ml in run for MCFA. The samples were injected in 'Eclipse Plus Phenyl-Hexyl, 4.6X100mm 3.5 micron' (Agilent Technologies) column. The parameters for HPLC system are,

Flow rate: 1ml/min

Wavelength: 250nm for SCFA and 210nm for MCFA

Run time: 15 min.

The results from peak shown in generated graph was taken and calculated by considering retention time and area with respect to it.

The composition of phosphate buffer was as given below,

Preparation for 1000ml,

1. Na_2HPO_4 = 11.1gm.
2. NaH_2PO_4 = 3.1gm.

PH for the buffer was 2.5

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