"Effect of Ketogenic Diet, Intermittent Fasting and High Carbohydrate Diet on Chronic Inflammation in Male Wistar Rats"

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By

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(Registration No: KLEU/Ph.D./15-16/DO1215004)

Under the Guidance of

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2021

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Date: ***Dr. Urmila Anil Kagal***

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LIST OF ABBREVIATIONS USED (IN ALPHABETICAL ORDER)

AcAc	:	Acetoacetate
Acetyl COA	:	Acetylcoenzyme A
AD	:	Alzheimer's disease
Apo A	:	Apolipoprotein A
Apo B	:	Apolipoprotein B
β – HB	:	Beta-Hydroxy Butyrate
CFA	:	Complete Freund's Adjuvant
CRP	:	C Reactive Protein
CVD	:	Cardiovascular Disease
DAMPs	:	Damage Associated Molecular Patterns
DM	:	Diabetes Mellitus
ETC	:	Electron Transport Chain
FFAs	:	Free Fatty Acids
HCD	:	High Carbohydrate diet
HDL	:	High Density Lipoproteins
IDL	:	Intermediate Density Lipoproteins
IF	:	Intermittent Fasting
IFNs	:	Interferons
ILs	:	Interleukins
IL-6	:	Interleukin-6
KBs	:	Ketone bodies
KD	:	Ketogenic diet
LDL	:	Low Density Lipoproteins

LPL	:	Lipoprotein Lipase
LPS	:	Lipopolysaccharides
MCTs	:	Mono Carboxylic acid Transporters
MDA	:	Malondialdehyde
NCDs	:	Non Communicable Diseases
PAMPs	:	Pathogen Associated Molecular Patterns
PRRs	:	Pattern Recognition Receptors
MAPKs	:	Mitogen Associated Protein Kinases
NF-KB	:	Nuclear Factor Kappa B
Nrf2	:	Nuclear Factor Erythroid-derived 2 (NF-E2)-related factor 2
NOXs	:	NADPH Oxidases
PD	:	Parkinson’s disease
RA	:	Rheumatoid Arthritis
ROS	:	Reactive Oxygen Species
SCI	:	Systemic Chronic Inflammation
SGC	:	Soluble Guanylate Cyclase
T2DM	:	Type 2 Diabetes Mellitus
TCA	:	Tricarboxylic Acid
TGs	:	Triglycerides / Triacylglycerols
TNF α	:	Tumor Necrosis Factor alpha
TPA	:	12-o-tetracanoilphorbol-13-acetate (TPA)
UCPs	:	Uncoupling Proteins
VLDL	:	Very Low Density Lipoproteins
WHO	:	World Health Organization

ABSTRACT

Background

It has been long recognized that diet is crucial in causation and management of chronic Non Communicable Diseases (NCDs). Chronic inflammation of mild to moderate intensity contributes to pathogenesis of several NCDs. Literature reveals that a diet rich in carbohydrate is a crucial risk factor in causation of NCDs. Hence a rational approach to diminish the risk of developing NCDs would be to reduce carbohydrate consumption by ketogenic diet (KD) or intermittent fasting (IF). Hence, the current study was undertaken.

Objectives:

To determine the effects of ketogenic diet, intermittent fasting, high carbohydrate diet and a combination of intermittent fasting with ketogenic diet on chronic inflammation (induced by Complete Freund's adjuvant) - (CFA) and oxidative stress parameters in male Wistar rats.

Methodology

Male Wistar rats were used and divided into six groups as follows: Group I (Control group) fed standard diet, Group II – Ketogenic diet, Group III – Intermittent fasting, Group IV - Intermittent fasting + Ketogenic Diet, Group V- High carbohydrate diet, Group VI - Normal group fed with standard diet. Respective diets were started four weeks prior to injection of CFA and continued throughout the study. CFA was injected into the right hindpaws of all groups except group VI. Volume of injected hindlimb was measured on several days. Ketone levels were measured at beginning and end of study. Other parameters measured were paw volume, cytokines,

oxidative stress parameters and histopathology of injected ankle joint. Statistical tests used were ANOVA and Post Hoc Dunnett's test. P value of < 0.05 was considered significant.

Results

It was found that, KD and IF alone and in combination can curb the inflammatory process by reducing paw edema, inflammatory markers and vulnerability to oxidative stress. As opposed to this current study also found that, carbohydrate rich food worsens inflammatory process triggered off by CFA.

Conclusion

Findings of current study point out that inflammatory process is modifiable by two dietary means both involving carbohydrate restriction namely intermittent fasting or consuming a ketogenic diet. Since carbohydrate forms a substantial chunk of most food that we consume, routine carbohydrate restriction by a ketogenic diet may not be practicable and intermittent fasting alone would be a more feasible alternative. Both modalities seem to have comparable potential in interfering with the progression of the inflammatory process.

Keywords: ketogenic diet; inflammation; carbohydrates; fasting**;** wistar rats

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1. INTRODUCTION

1.1 Background

It has been recognized from time immemorial that diet is crucial in causation and management of disease – especially in chronic Non Communicable Diseases (NCDs). The World Health Organization (WHO) states that Non Communicable Diseases kill around seventeen million persons before they reach seventy and forty million persons every year. This accounts for 70% mortality the world over. Twenty years hence NCDs will amount to countries the world over having to spend trillions of dollars on management of these diseases. NCDs are already thrusting populations into becoming monetarily poor. There has been over forty years, a paradigm shift in global disease trends. There was a time when illnesses of infectious nature were predominant in countries with middle and low level incomes. The scenario has now drastically changed with NCDs having overtaken infectious diseases and posing a threat to the developed and developing world. The burden of NCDs like cancer, diabetes, obesity and coronary artery disease has superseded malnutrition, tuberculosis, malaria and HIV.[1, 2]

Inflammation is crucial for host defence. But, long drawn inflammation of mild to moderate intensity in the recent past has been recognized by scientists as disastrous. A variety of NCDs namely diabetes mellitus (DM), obesity, ischaemic heart disease and fatty liver (not caused by chronic alcoholism) are found to be harbouring long standing mild inflammation contributing to their pathogenesis.[3]

Inflammation with its cardinal features of swelling, redness, etc may be beneficial during short periods in helping tissues heal. Metabolic disorders mentioned previously are evidence to the fact that, inflammation in the long term is not helpful. Mediators of classic inflammatory response and those observed in disorders of metabolism might be the same.[4]

Factors such as migration to cities as well as increased advertising and affordable prices of junk food are causing increased intake of refined grains, sweet beverages and bakery products which enhance the possibility of developing NCDs. The current lifestyle is exactly opposite to that of our forefathers who were physically fit and robust as they were hunter gatherers and used to go on foot covering huge distances in their search for food.[2, 5, 6]

Vulnerability to NCDs depends on a complex combination of genes and influences in the environment. Developing economies have hordes of people migrating from the villages to dwellings in the city due to which their style of living and food habits have changed drastically in comparison to their lives in the villages. These factors have made them susceptible to NCDs.[7] The Indian Government has put forth a programme for control of NCDs as a result of the growing encumbrance caused by them. According to the programme, diet as a form of lifestyle forms the foundation for prophylaxis as well as management of NCDs.[8]

Studies done so far have evaluated the effect of ketogenic diet (KD) and fasting on inflammation in the short term. Vasconcelos et al and Dupuis et al have evaluated effects of intermittent fasting (IF) and KD respectively on lipopolycsaccharide (LPS) induced inflammation which has been evaluated forty eight hours later through its effects on behavioural tests, proinflammatory cytokines

and neurotrophic factors.[9,10] Wan et al evaluated effects of IF on parameters like size of the ischemic infarct and inflammatory markers twenty four hours after irreversible coronary artery ligation.[11] In all these studies IF and KD were found to improve the predefined parameters.

Ruskin et al investigated the relationship between KD and local inflammatory response triggered by Complete Freund's Adjuvant (CFA) in rats. The study revealed that, swelling and plasma extravasation in the rat hindpaws were significantly lower in KD treated groups.[12]

In a study by Wang et al spinal cord injury was induced in rats to evaluate impact of ketogenic metabolism on antioxidant activity. It was done with the help of dietary intervention. The study found that, in the dietary intervention groups, ketogenic metabolism caused a rise in anti-oxidative stress genes namely Foxo3a and Mt2.[13]

Razeghi Jahromi et al investigated role of fasting on Experimental Autoimmune Encephalomyelitis in mice and found that, IF suppressed the secretion of inflammatory cytokines and raised IL-10 (interleukin -10, an anti-inflammatory cytokine) production in splenocytes .[14]

The literature studied so far reveals that a diet rich in carbohydrate is a crucial risk factor in causation of NCDs and that long standing inflammatory process underlies the pathogenesis of NCDs. Hence an economical and rational method to diminish the possibility of developing such diseases would involve reducing carbohydrate consumption by KD or IF. Hence, the current study was undertaken with an objective of determining roles of ketogenic diet, intermittent fasting and high carbohydrate diet (HCD) in inflammatory process stimulated by CFA in male Wistar rats.

LITERATURE REVIEW

1.2.1 History relevant to fasting:

The history of fasting can't have a starting point because man has been practicing fasting since time immemorial. Even animals fast when they are ill and sometimes when they feel even slightly uneasy. Ancient Greek philosophers and healers like Hippocrates, Socrates and Aristotle believed in the benefits of fasting. Fasting is practiced by religions across the world including Christians, Jews, Hindus, Buddhists and Muslims for the purposes of mourning and purifying the soul, as a form of sacrifice and preventing gluttony.

Muslims all over the world fast during Ramadan from sunrise to sunset which lasts for one month. They eat after sunset and just before sunrise. Greek Orthodox Christians fast for 180 - 200 days every year. There are three fasting periods namely Nativity, Lent and the Assumption.

In Hinduism, upavas or fasting holds great significance. It is an essential part of Upasana (worship). It is considered a form of self-purification and a means of attaining spiritual vigor (tejas), earning merit (punya), a way of pacifying furious gods or as a means of washing away one's sins. Most Hindus fast during the month of Shravan (July / August). People sometimes fast on specific days of the week, for instance on Monday to please Lord Shiva, on Friday which is considered ideal to please most goddesses and so on.[15,16]

1.2.2 History of Ketogenic Diet:

Two crucial observations were made in 1921. Firstly, Woodyatt observed that ketone bodies (KBs) namely beta-hydroxybutyric acid and acetone are physiological during starvation or consumption of a diet low in carbohydrate and high in fat. Concomitantly, in the US, Dr. Wilder said that KD offers all the advantages of fasting and also produces a state of ketonemia. Dr. Wilder for the first time used the word "ketogenic diet". Subsequently Dr. Wilder and his co -workers started to explore the possible use of KD in incurable epilepsy and observed that a large number of cases benefitted. At the beginning of the 18^{th} century, a diet low in carbohydrate became extremely popular for diabetes because insulin had not yet been discovered. In 1972, Dr. Atkins popularized this concept with the common man as an approach to weight loss.

1.2.3 Introduction to Ketogenic Diet and Types of Fasting:

Ketogenic diets are those where in carbohydrates are drastically reduced to lower than 50 g/day. Further such diets have an increase in protein and fat content.

Fasting can be of the following types:

i. Restriction of calories where in person's everyday intake of calories is brought down by 20–40%, but the number of meals remain the same;
ii. Intermittent fasting where in there is eliminating food intake intermittently;
iii. Restriction of feeding which is time based – where in person eats strictly during a four to six hour period every day.

KBs such as acetoacetate (AcAc) and betahydoxybutyrate are produced as a result of fat oxidation. This happens when fats start getting utilized for fuel instead of glucose. Variations of KD exist.[17]

Both KD and fasting induce a state of physiological ketosis. This term was coined by the famous biochemist Hans Krebs. In physiological ketosis, ketone levels in the blood do not exceed 7-8mmol/litre and the pH of blood does not change.[18,19]

1.2.4 Ketone metabolism

Three compounds constitute KBs. They are: acetoacetate, β-hydroxybutyrate (βHB) as well as acetone. Concentrations in blood range from 0.1mM following meals, 6mM in prolonged state of caloric restriction and 25mM in uncontrolled diabetes.

KBs are synthesized as a result of Acetyl coenzyme A (acetyl-CoA) excess. This process is called ketogenesis. Acetyl-CoA in the mitochondria of the liver serves as the source for ketogenesis.

Ketones are utilized mainly by heart and kidney. Circulating fatty acids cannot be utilized by the brain as a source of energy. The ketogenic pathway (a fat derived fuel) provides the greatest evolutionary advantage by serving as a chief caloric source for brain during periods of low food availability.[20]

In the vast majority of mammals, liver stores glucose as glycogen. In man, based on physical activity, twelve to twenty four hours of fasting will culminate in low blood glucose and exhaustion of glycogen from the liver. In such a situation, glucose from sources other than the liver, ketones and fats are utilized as fuel.[19]

Normally, acetyl-coA from the liver enters Krebs cycle. Acetyl Co-A is shifted to generate ketone bodies during periods of high fat levels which cannot be handled by tricarboxylic acid (TCA) cycle. Acetoacetyl-coA serves as a precursor for ketone synthesis. Acetoacetyl-CoA is the product of two acetyl-CoAs combined in the presence of thiolase. Acetone generated from acetacetate is thrown out as a volatile substance from lungs and kidneys.[21,22]

In blood, acetacetate and βHB are carried by monocarboxylic acid transporters (MCTs) to the interstitial space of the brain, glia and neurons. Within the mitochondria of the neurons, acetacetate and βHB transform themselves to acetyl-CoA through a cascade of reactions. Two acetyl-CoA molecules enter the Krebs cycle.[21,22]

The liver synthesizes ketones. The chief hepatic KB is acetoacetate, but βHB circulates in the blood. βHB being a biochemically stable compound is transported to the tissues. In the tissues βHB reverts to acetoacetate, which forms acetoacetyl-CoA. Ultimately, acetyl-CoA is utilized in the TCA cycle. Presence of circulating ketone bodies causes ketonemia. Ketonuria results from their presence in urine.[23,24,25]

1.2.5 Definition of Lipids

Lipids are actually unrelated compounds but are related by virtue of their physical characteristics namely water insolubility and dissolution in compounds like ether and chloroform.[26,27]

1.2.6 Functions of Lipids:

i. Fats and oils are reservoirs of energy in living beings. Fat also serves as an insulator against cold.

ii. Phospholipids and sterols function as crucial structural elements of biological membranes.

iii. Other lipids play major roles as emulsifiers in the gut. They assist in folding of membrane proteins. They may act as messengers inside the cells.

iv. Lipids act as eicosanoids which play crucial roles as prostaglandins and leukotrienes

v. Triacylglycerols are lipids stored in adipose tissue.

vi. Phosphoacylglycerols are components of lipoproteins, lung surfactant, precursors of second messengers and nervous tissue constituents.

vii. Glycolipids are important constituents of brain and cell membrane.

viii. Cholesterol constitutes a vital chunk of membranes and is the precursor of steroids, Vit. D and bile acids.[26,27]

1.2.7 Lipid Classes:

Lipids are termed as simple or complex based on whether they are made up of a combination of fatty acid esters with alcohols or contain functional groups in addition to fatty acid esters and alcohols. Simple lipids are sub-classified into fats, oils and waxes. Complex lipids are sub-classified into phospholipids, glycolipids, sulfolipids, aminolipids and lipoproteins.[26,27]

1.2.8 Fatty Acid Classes:

Fatty acids are classified based on the absence or presence of carbon double bonds as saturated and unsaturated respectively. Further, fatty acids based on carbon double bond count are classified as monounsaturated and polyunsaturated. Eicosanoids are derived from twenty carbon containing fatty acids.[26,27]

1.2.9 Lipids of Physiological / Clinical Significance:

Steroids:

All steroids have a cyclic nucleus connected to a cyclopentane ring. Since cholesterol is associated with atherosclerosis it is probably the best known steroid. Cholesterol is a precursor of various hormones, Vit. D and bile acids.[26,27]

Micelles:

Most lipids contain a predominance of nonpolar (hydrocarbon) groups and hence are insoluble in water. But some micelles may have water soluble groups making them amphipathic in nature. Micelles play a vital role in fat absorption from the gut.[26,27]

Triglycerides (TGs):

They are glycerol esters joined to fatty acids. Mono- and diglycerides help in in formation and breakdown of triglycerides.[26,27]

Phospholipids

Phospholipids are derived from phosphatidic acid. Phosphatidic acid ultimately forms cardiolipin, a major lipid of mitochondrial membranes. Phospholipids in cell membranes are mostly phosphoacylglycerols with a choline component. Choline gives rise to acetylcholine which is vital in neurotransmission[26,27]

Sphingomyelins:

Sphingomyelins are crucial in the nervous system. Ceramide is an type of sphingomyelin.[26,27]

Glycolipids:

Glycolipids are widely distributed all over the body but are particularly well distributed in the brain. They form a component of carbohydrates present on cell membranes. Animals contain mainly glycosphingolipids. Gangliosides are complex glycosphingolipids present mainly in nerve cells.[26,27]

1.3 Generation of fatty acids:

Dee novo generation of fats is called lipogenesis. This process occurs in cytosol. The immediate substrate is Acetyl- CoA. The first step in this process also happens to be the controlling step which is the production of malonyl-CoA from acetyl coA where bicarbonate and ATP are required. Enzyme needed is acetyl-CoA carboxylase. The chief source of NADPH for fat synthesis is the pentose phosphate pathway. Acetyl-CoA is the precursor the fatty acids generated from glucose. The process of lengthening of fatty acids takes place in endoplasmic reticulum through the

microsomal system. The pathway uses malonyl-CoA as acetyl donor and NADPH as reductant.[26,27]

1.3.1 Role of the Liver in Lipid Metabolism:

Liver is the epicenter of fat metabolism. The liver has the functions listed below:

i. It synthesizes bile, which contains cholesterol and bile salts. Bile facilitates lipid digestion and absorption.
ii. Liver has enzymes for generating TGs and phospholipids. Liver enzymes also involve in fatty acid generation and oxidation.
iii. Liver is the site of ketone synthesis.
iv. Liver plays a major role in lipoprotein generation and breakdown

Lipid metabolism is required to transport lipids to the periphery for utilization and also lipid delivery to the liver for removal and regeneration. Fatty acids in their free form (FFA) are the most metabolically active lipid fraction.

1.3.2 Lipoproteins

The gut and liver secrete lipoproteins which carry lipids to the periphery. They also return them to the liver for breakdown and regeneration. Lipoproteins are made up of a water insoluble centre and are surrounded by an amphipathic component. TGs and esters of cholesterol are at the centre. The protein component is apolipoprotein. TGs are present mainly in chylomicrons and very low density lipoproteins (VLDL). Cholesterol is present mainly in low density lipoproteins (LDL). Phospholipid is the main component of high-density lipoproteins (HDL).

Lipoproteins are grouped into chylomicrons, VLDL, LDL and HDL.[26,27]

1.3.3 Pathways of lipid metabolism:

Three pathways exist: Exogenous which deals with food derived lipids. Endogenous which deals with handling of hepatic lipids. Reverse cholesterol transport takes care of transporting cholesterol from distant sites to liver.

i. **Exogenous pathway:**

This involves packaging of fats derived from meals into chylomicrons. Chylomicrons are derived from chyle of the intestinal lymph. They transport food derived lipids into the blood. These chylomicron packages after being unleashed into the lymph and further into the blood acquire various apolipoproteins. Lipoprotein lipase (LPL) break down TGs and unleashes fatty acids for cell uptake. Chylomicron fragments are thrown into the circulation which are taken up by hepatic receptors where they are endocytosed.[28,29]

ii. **Endogenous pathway**

In this pathway, the liver synthesizes VLDL packages. VLDLs contain TGs and cholesterol assembled with apolipoprotein B (ApoB). VLDLs are triglyceride transporters from liver to periphery being broken down for cellular uptake by LPL in fat tissue. Intermediate density lipoproteins (IDLs) are broken down by hepatic lipases into LDLs. LDLs are the ligands for specific receptors in the liver and gut. LDLs are broken down and cholesterol is released. Remaining LDLs can be converted to Lipoprotein A by binding to free apolipoprotein A (ApoA) secreted by the liver. LPL is present in tiny blood vessels of various tissues. It breaks down lipoproteins anchored to the endothelium. Triacylglycerol is broken down to its core components.[28,29]

iii. **Reverse cholesterol transport:**

This pathway transports cholesterol from periphery to liver. This pathway is vital in cholesterol homeostasis. The liver and intestines give out apolipoprotein A –I (ApoA-I) lacking in lipids. ApoA-I acquires lipids during cholesterol return from the periphery and macrophages. Cholesterol attainment results in ripe HDLs which occurs though an HDL cycle. Cholesterol is taken away from HDL by the liver. Fat deprived HDL is broken down or returned to the blood for reacquiring cholesterol.[26,29]

1.3.4 The Carnitine Shuttle

Oxidation of fats in animals takes place in the mitochondria. Fats having a chain of more than twelve carbons need transportation through proteins. This is the case with most fats derived from diet or those unleashed from adipose tissue. The carnitine shuttle involves complex biochemical reactions which help lengthy fatty acids to reach matrix of the mitochondria for oxidation.[27]

1.3.5 Fat oxidation:

In β-oxidation, starting at the carboxyl end, two carbon atoms are removed from acetyl-CoA. A series of reactions occur in the mitochondria resulting in cleaving of fatty acids ultimately releasing acetyl-CoA. Electron carriers generated through this process give electrons to the respiratory chain. These electrons are used in adenosine triphosphate (ATP) generation.[27]

1.3.6 Introduction to Carbohydrates

Carbohydrates are organic compounds having carbon, hydrogen and oxygen.[26,27] They provide energy to living organisms.

1.3.7 Classification of carbohydrates:

Carbohydrates which can be digested include monosaccharides, disaccharides and some polysaccaharides. Fiber cannot be digested.

i. Monosaccharides:

They are carbohydrates in their simplest form which are not amenable to break down. The number of carbon atoms in their structure can vary from three to nine. Hence, we call them as trioses, tetroses, pentoses and so on. They can also be classified based on the type of carbonyl group into aldose and ketose.

ii. Disaccharides:

Sugars with 2 monosaccharides united by a glycoside the usual ones being maltose, lactose, and sucrose. Other disaccharides present in nature are trehalose, cellobiose, and gentiobiose.

Lactose occurs in milk and its products.

Sucrose contains glucose and fructose.

iii. Oligosaccharides:

Consist of 2 to 10 monosaccharides. They are not amenable to cleavage by the gut.

iv. Polysaccharides (also called glycans)

They are high-molecular-weight polymers of monosaccharides. They contain greater than 10 monosaccharides. Chief polysaccharides are cellulose, starch and glycogen. [26,27,30,31]

1.3.8 Digestion of Carbohydrates

Carbohydrate breakdown begins with α amylase of saliva. The α-1,4 glycosidic bonds are broken down by α amylases of the saliva and pancreas. Thirty to forty percent of carbohydrate breakdown occurs before reaching the intestines. In the small bowel, α -amylase of the pancreas cleaves carbohydrates. Additional cleavage of starch is needed before it is absorbed. This is done by the disaccharidases which are brush-border enzymes of intestinal epithelial cells (enterocytes). After the breakdown of various carbohydrates into monosaccharides they enter the intestinal epithelial cells. Entry into intestine requires transporters namely SGLT1, GLUT2 and GLUT5. The neighboring circulation takes up sugars from the enterocytes.[32]

1.3.9 Pathways of Carbohydrate Metabolism

i. Glycolysis:

Glycolysis is glucose breakdown to pyruvate. This occurs in most mammalian tissues and is a major source of ATP. Glycolysis takes place in cytoplasm of all cells. Its peculiarity is that it can occur with or without oxygen. Main catalysts are hexokinase, phosphofructokinase and pyruvate kinase. 5 steps in the beginning are preparatory phase which involves using ATP to increase the energy of the in between compounds. Energy is generated in the payoff phase. Ultimately two ATPs are generated from every glucose utilized. Acetyl–coA enters the Krebs cycle. The electrons are used for ATP synthesis in the mitochondria. Pyruvate can also be converted to lactate in exercising skeletal muscle where ATP synthesis is urgently needed. Glycolysis in erythrocytes, always terminates in lactate because they don't have mitochondria. Other tissues generating lactate are brain, gut and retina.[26,27]

ii. Citric Acid Cycle or Krebs Cycle or Tricarboxylic Acid (TCA) Cycle brings about oxidation of acetyl- CoA.

a. It starts with acetyl-CoA forming citrate.

b. Citrate converts to isocitrate which in turn yields α-ketoglutarate

c. Succinate is a derivative of α-ketoglutarate.

d. Succinate converts to oxaloacetate which reacts with acetyl-CoA.

In the citric acid cycle energy is preserved in the form of NADH and FADH2. [26,27]

iii. Glycogenolysis

This is the process of glycogen breakdown which results in glucose synthesis in liver and lactate generation in muscle as a consequence of presence and absence of glucose-6-phosphatase respectively. Glycogen phosphorylase, glucan transferase and glucose-6-phosphatase are various enzymes involved in glycogenolysis leading ultimately to complete glycogen breakdown. In hepatic and renal tissue, but not in muscle, glucose-6-phosphatase, which cleaves glucose 6-phosphate exists. Presence of this enzyme yields glucose which is transported causing rise in blood glucose.

iv. Gluconeogenesis:

Gluconeogenesis refers to all paths where in various sources other than carbohydrate are converted to glucose or glycogen. Such glucose precursors are generated in liver or are taken to the liver from the periphery via the bloodstream. Glycogenolysis helps maintain blood glucose during short period of caloric restriction. During long periods of caloric restriction glycogen is exhausted and liver cells generate glucose by gluconeogenesis. This process requires lactate, pyruvate, glycerol and protein precursors.[26,27]

1.4 History of inflammation:

Celsus in first century first described the clinical symptoms of inflammation. These features are today considered pathognomonic of inflammation. These are redness, swelling, raised temperature and pain. The underlying mechanism was explained by Waller and Cohnheim. The 5th feature, namely loss of function, was described by Virchow. Phagocytosis was first observed by Metchnikoff.[33]

1.4.1 Process of Inflammation:

Inflammation typically is made up of inducers, detecting sensors and mediators which afflict various targets in the body. The inducers are infecting microorganisms or are pointers from dead cells and injured tissues, toxins and foreign bodies. Sensors are molecules being stimulated by inducing substances. These sensors induce mediator synthesis. The mediators are chemicals synthesized within the body. They activate tissues and cells and encourage or prevent inflammation and tissue repair. Inducers are PAMPs from microorganisms or DAMPs secreted by damaged tissues. PAMPs are pathogen associated molecular patterns. DAMPs are damage associated molecular patterns. These patterns are acknowledged by different receptors on the inflammatory cells. On stimulation of these receptors, various inflammatory cytokines are released. They bring about variations on the endothelium such that, inflammatory cells can pass through the intersections of endothelial cells.[33, 34, 35]

Although the body may respond differently based on the type of inflammatory stimulus and its exact site, the inflammatory response processes share a common mechanism, which are abridged as follows:

i. Cell surface pattern receptors acknowledging dangerous stimuli

PAMPs from microbes set off inflammation by activation of receptors termed as pattern recognition receptors (PRRs). These PRRs are located on cells of immunity as well as others. Some of these receptors acknowledge DAMPs which are biomolecules in host which trigger off and preserve an inflammation of noninfectious nature.[36,37,38]

ii. Pathways of inflammation

These pathways affect the genesis of a variety of long term illnesses. Intermediaries of inflammation and pathways involved are common to all diseases. Stimuli trigger off signaling cascades within cells. These in turn induce generation of intermediaries of inflammation. Main stimuli are microorganism derived products and various cytokines which intervene in inflammation by interacting with receptors. Receptor stimulation sets off cellular cascades which include JAK-STAT, mitogen associated protein kinases (MAPKs) and nuclear factor kappa-B (NF-κB) pathways.[36,39]

NF-κB controls inflammation, immunity, existence and apoptosis. It also controls production of cytokines which favour inflammation.[40] MAPKs are a group of enzymes containing serine/threonine. They mediate reactions of cells to various stressors which control cell multiplication and death.[41]JAK-STAT pathway has been preserved and involves diverse cytokines, growth factors and interferons. It controls deoxyribonucleic acid (DNA) expression through factors outside the cell. JAKs are stimulated by ligands. They provide anchorage for STATs which are cytosolic

transcription factors. After activation they move to the nucleus. This pathway causes direct conversion of a signal outside the cell into a DNA mediated response.[42]

iii. Release of markers of inflammation:

Markers are utilized clinically to indicate presence of a pathological process. They are also used as a marker to know whether therapy is working. These markers predict presence of a host of diseases of inflammation such as diseases of heart, blood vessels and infection. These markers are generated by inflammatory cells.

a. C reactive protein (CRP):

CRP is called so as a result of its binding capacity to polysaccharide (component C) of Streptococcus pneumoniae. CRP helps detect presence of extraneous pathogens containing phosphocholine. Also helps detect injured cells having phospholipids. CRP can also hasten disposal of dead cells. CRP also activates complement. It can rise thirty times from baseline (<5 mg/L) during acute inflammation. It helps monitoring patients with rheumatoid arthritis and Crohn's disease. Its sensitivity and specificity is greater than ESR. Levels of CRP start rising approximately six hours after the setting in of inflammation and peak after forty eight hours. It starts to fall if the inciting trigger remits. Inflammation favouring consequences of CRP are complement activation apart from recruitment of monocytes synthesizing cytokines. CRP at times can exacerbate tissue injury. Elevated CRP is an undisputable indication of ongoing inflammation.[43,44]

b. Cytokines:

They are tiny proteins released by cells regulating growth and development. They also control responses of specific cells. Cytokines have a molecular weight of 5 to 30 kDa. They are extremely short lived. Their local content is measured in picomoles. Cytokines may be named as interleukins (ILs), interferons (IFNs) and so on. They are produced by cells of immunity and some others as well. They bind to receptors and control gene expression. They may act locally and systemically.[45]

c. Other inflammatory markers:

They are reactive oxygen species (ROS), malondialdehyde (MDA) and isoprostanes. They trigger off NF-κB, p53, and STAT.[46]

1.4.2 Chronic Inflammation:

Systemic chronic inflammation (SCI) is set off by DAMPs. This happens when there is no acute injury to the cell. The inflammation is of a low level and relentless in nature. It finally causes damage over a long period.[47]

1.4.3 Factors predisposing to SCI:

i. Infections:

Infections lasting a lifetime such as cytomegalovirus, hepatitis C virus, etc. have been found to have association with autoimmune diseases, cancers, neurodegenerative diseases and cardiovascular disease (CVD). But this is a matter of debate. The influence of chronic infections on chronic diseases appears to depend on the environment and underlying gene makeup.[47,48,49]

ii. Physical inactivity:

Skeletal tissue has been found to produce cytokines and myokines which have systemic effects. This secretion takes place in a contracting muscle which is said to reduce inflammation systemically. Low physical activity is directly associated with opposition to anabolism, raised CRP and high cytokine levels in normal persons and T2DM patients. This in turn results in insulin opposition, dyslipidemia and hypertension. These conditions increase the risk of CVD, T2DM, cancer and Alzheimer's disease in chronically inactive people.[47,50,51]

iii. Psychological stress:

Persistent psychological stress disrupts glucocorticoid anti-inflammatory function leading in turn to SCI and poor health. Contact with blue light after sunset and increased nocturnal activity cause malfunctioning of circadian pathways in night-shift workers which promotes inflammation and predisposes such individuals to metabolic syndrome and cancers of breast, ovary, prostate, colorectum and pancreas.[47,52,53]

iv. Dietary factors:

Dietary factors contributing to alteration of the composition and function of gut microbiota are foods high in refined grains, sugar, flavour additives, ultra-processed foods, alcohol and foods containing emulsifiers. Alteration of gut microbiota is associated with raised intestinal leakage and immunological changes of epigenetic nature. Ultimately low-grade endotoxemia and SCI sets in. Also, there are vital cellular metabolic changes and increase in generation of and inefficient clearance of malfunctioning mitochondria and oxidized molecules present within the body. These defective components rise with progression of age and are acknowledged as

DAMPs. This leads to activation of the inflammasome machinery and amplified inflammatory response. Ultimately a biological state termed as "inflammaging," develops which is the result of prolonged physiological activation of innate immune system.[47,54,55]

1.4.4 Role of inflammation in noncommunicable diseases (NCDs):

i. Role of inflammation in atherosclerosis:

Atherosclerosis is a state of systemic inflammation where in there is an increased plasma level of mediators of inflammation. Also there is malfunctioning of the endothelium resulting in less vasodilation mediated by endothelial cells. An injured endothelial tissue accumulates cholesterol rich LDL. These LDL particles in walls of blood vessels get oxidized and trigger an inflammatory response which fails to resolve. This is the origin of atherosclerosis. The immune system contributes to early malfunctioning of endothelium to progression of the disease to the stage of development of sudden thrombotic events. These events are the consequence of plaque breach. Monocytes convert to macrophages and finally to foam cells. These cells form the dead centre of the atheromatous plaque. Activated macrophages release inflammation favouring cytokines. These cytokines are major drivers of atherosclerosis. Enhanced production of inflammatory mediators causes intramural inflammation. These intermediaries can also reach the bloodstream and affect microcirculation. Also, underlying factors for CVD such as hypercholesterolemia, high blood pressure, T2DM and overweight can induce malfunctioning of the endothelium in the microcirculation.

Oxidative stress is vital in initiating and advancing atherosclerosis. Superoxide anions and hydrogen peroxide which have remarkable oxidizing activity are produced by the vascular endothelium and smooth muscle as well as macrophages.[56,57,58,59,60]

ii. **Neurodegenerative disorders:**

Neurodegenerative disorders are a consequence of the amalgamation of exposure to toxins in the environment and defective genes. Oxidative stress also makes a significant contribution. Damage to DNA and RNA are considered an essential feature of these diseases.[61]

a. **Alzheimer's disease (AD):**

AD is a long term inflammation of the nervous tissue in the brain. Stimulated microglia are found in AD afflicted areas as also around amyloid plaques. These findings have been revealed by postmortem studies of brains of AD patients. Raised levels of ROS in brain and CSF of AD patients support a hypothesis that, ROS contribute to cell death and neurodegeneration. Further, oxidative stress can induce strand breaks and large deletions in DNA, leading to DNA damage. Also, elevation of serum CRP and IL-6 are found in these patients five years prior to presentation with clinically significant deranged mentation.[59,62,63,64]

b. Parkinson's disease (PD):

Depletion of glutathione in substantia nigra is an early development detectable in the brain of PD patients. A major hallmark of PD is reduced complex I functioning of the mitochondria. This is a result of glutathione deficiency in dopaminergic neurons and there is an accompanying marked reduction in mitochondrial function.

Oxidative stress in PD has been demonstrated in studies proving that oxidative stress induces nigral cell degeneration.[65]

iii. **Rheumatoid arthritis (RA):**

In RA, synovium is inflamed and this is a reaction to synovial cell hyperplasia, excess of synovial fluid and pannus formation. Disease progression finally causes degeneration of articular cartilage and pathological fusion of the joints. Apart from this, lungs, pericardium and eyes can be affected. There is also development of nodules in the subcutaneous tissue. The ambiance of inflammation of synovium is controlled by a complicated network of cytokines. Cytokines induce and worsen inflammation by stimulating the endothelium and drawing cells of immunity towards the synovium.[59,66,67]

1.4.5 History of Free Radicals:

Moses Gomberg gave the first description of free radicals more than a century ago. Their presence in biological systems was not considered for a very long time. Leonor Michaelis after a very long time hypothesized that, that all oxidative reactions which involve living tissue are controlled by free radicals. Although an incorrect hypothesis, it triggered off research of the part played by free radicals in living tissues. A 2nd epoch of free radical research in biological systems was the discovery of superoxide dismutase a protective enzyme against free radicals by McCord and Fridovich. In 1977 came the third era of free radical research where in Mittal and Murad proved that hydroxyl radical, triggers off the activity of guanylate cyclase.[68,69]

1.4.6 Introduction:

Free radicals are defined as substances having a considerable degree of reactivity as a result of having unpaired electrons within them.[69] ROS are metabolites of oxygen which are in a state of partial reduction and possess strong oxidizing capabilities. At low levels they function as signaling molecules controlling physiological functions such as cellular growth and maturation, ageing and programmed cell death. At high concentrations, they are deleterious to cells as a result of their ability to damage DNA, protein and lipoidal cellular constituents.

The biological ROS are molecules which last for a short period and have unpaired electrons such as perhydroxyl radical ($HOO^{\cdot}$), superoxide anions and hydroxyl radical ($HO^{\cdot}$). Nonradical ROS are hydrogen peroxide (H_2O_2), aldehydes, hypochlorous acid (HOCl) and ozone.[70]

1.4.7 Origins of ROS:

ROS originate extraneously or from within the body.

i. Origins within the body:

a. A wide variety of biochemical and physiological processes give rise to ROS. Among the different enzymes which have been implicated in ROS generation are the 7 types of transmembrane NADPH oxidases (NOXs) which generate superoxide. The cytosolic components of NOXs bring about electron transfer. The electron is ultimately handed over to oxygen on the outer side of a cell bringing about the generation of superoxide.

b. An important generator of ROS are the mitochondria. Mitochondria generate superoxide at 2 distinct locations in the electron transport chain (ETC), namely Complexes I and III upon the transfer of one electron to oxygen. Semiquinone anion species ($Q^{\cdot -}$) is a nonenzymatic source of ROS in mitochondria. It is generated during the recycling of coenzyme Q_{10}. After its formation the semiquinone anion species hand over electrons to O_2 bringing about synthesis of superoxide.

c. Other cellular sources of ROS production are enzymes within the endoplasmic reticulum, lipoxygenases, cytochrome p450 enzymes and nitric oxide synthases.[71]

ii. Extraneous origins of ROS:

Sources from the environment are UV and ionizing radiation as well as toxins. Toxins such as paraquat react to form peroxides or ozone; superoxide formation promoting chemicals are quinones, nitroaromatics and bipyrimidiulium herbicides.[71]

1.4.8 Physiological Roles of ROS:

i. Role in Respiratory Burst:

On coming into contact with or swallowing bacteria or any exogenous material, stimulated phagocytes tend to generate huge amounts of superoxide and hydrogen peroxide. This phenomenon is called respiratory burst. There is involvement of myeloperoxidase, which catalyzes oxidation of halide ions. This reaction yields dominant oxidizers such as hypochlorous acid and xanthine oxidase. These oxidizers are vital in dealing with pathogenic organisms.[69]

ii. Apoptosis:

Apoptosis is cell death occurring in an organized manner which is vital to normal maturation as well as demolition of cells posing danger to the veracity of an organism. Whether a cell should kill itself or not is decided by a fine tuning between withdrawal of positive stimuli and delivery of negative stimuli. These mechanisms set off within the cell are represented by destruction of cell by ROS, irradiation, etc. which in turn stimulate Bcl-2 and ultimately creating holes in the mitochondria. These holes stimulate cytochrome c to be released which in turn utilizing ATP derived energy, activates a cascade of reactions which finally kill the cell.[69,72]

iii. Role in cell adhesion:

Cell adhesion has a crucial part to play in embryonic development, growth and maturation and injury repair. Variations in the adhering abilities of cells are strictly controlled by redox reactions. Various cytokines and bacterial lipopolysaccharides stimulate expression of cell adhesion molecules. ROS induce sticking of white blood cells to the endothelium.[69,73]

iv. Regulation of vascular tone:

Control of tone of blood vessels by cGMP is a case in point. Soluble guanylate cyclase (sGC) is stimulated through hydrogen peroxide and nitric oxide radical. cGMP produced by sGC regulates activity of protein kinases and ion channels. Vital functions controlled by nitric oxide are smooth muscle tone and suppression of platelet adhesion.[69,74]

1.4.9 Antioxidants:

Antioxidants are defined as small molecules which have any one of the following actions on free radicals:

They react with and reduce them

They forage them

They inhibit their generation or

They interfere with their activity[75]

Because free radicals are capable of causing widespread destruction within the body, a wide variety of antioxidants within and outside the body, exist to protect the cell. There are 3 antioxidant groups: enzymes, chain breakers as well as transition metal binding proteins.[76]

1.5 Antioxidant Enzymes:

i. Catalase:

The discovery of catalase in the 19th century by Thenard was associated with his discovery of hydrogen peroxide. The origin of catalase is the result of tissue degradation of hydrogen peroxide in the living. Catalase was named by Loew. Following phylogene analyses catalases are segregated into Clade 1, 2 and 3. Catalase catalyses formation of H_2O and O_2 from H_2O_2.[77]

i. Superoxide dismutases (SODs):

SODs are enzymes playing a crucial part in degradation of superoxide. The name superoxide dismutase has been earned by them because of their capability to

convert two superoxide moieties to 1each of dioxygen and hydrogen peroxide. In the process, SODs consume 2 equivalents of hydrogen ions. H_2O_2 is catalyzed by catalase or glutathione peroxidase. They avert damaging oxidizing chain reactions and prevent generation of H_2O_2, hydroxyl radical, peroxynitrate as well as hypochlorite.[78]

Types of SODs are SOD1 (cytosolic Cu/ZnSOD), SOD2 (MnSOD) and SOD3 (Cu/ZnSOD). Tissues containing SOD are mainly blood vessels, lungs, kidney and heart.[79]

ii. Glutathione peroxidases and Glutathione reductase (GR)

Glutathione peroxidases catalyse conversion of glutathione into its oxidative form using hydrogen peroxide or a lipid hydroperoxide as a substrate. Glutathione peroxidase has a plasma form generated in kidney. Vast amounts within the cell are found in the liver. The cytosol and mitochondria are the cellular components where glutathione peroxidase is predominantly distributed. It is the chief destroyer of hydrogen peroxide. Glutathione peroxidase is dependent for its activity on reduced glutathione. GR maintains a high proportion of reduced glutathione.[76,80]

1.5.1 Chain Breakers

Interaction between a free radical and a different molecule may generate secondary radicals. These in turn attack various targets to produce some more dangerous products. Chain breakers antioxidants take or give an electron from or to radicals respectively forming stable byproducts. Examples of such antioxidants are ascorbate, Vitamin E, flavonoids, Ubiquinol-10 and proteins such as ferritin, transferrin and caeruloplasmin.[81,82]

1.5.2 Pathological Role of ROS:

Oxidative stress refers to a phenomenon where in ROS generation is beyond the capability of the cell to synthesize antioxidants. There is an excess of ROS generation and hence protection by antioxidant is limited. Oxidative stress is damaging to cell components such as fatty acids and DNA apart from others. This has been responsible for contributing to diseases such as CVD, cancer and neurodegenerative disorders.[68,69]

1.5.3 Animal Models for Testing Anti-inflammatory Agents: [83,84,85,86,87]

i. Procedures for examining acute and subacute inflammation:

The acute phase of inflammation shows local blood vessel dilatation and capillary leakage. The subacute type shows infiltration by phagocytes. The following are the pharmacological techniques for testing these types of inflammation:

a. Vascular permeability:

One of the features of inflammation is increased vascular permeability. The model elaborated upon below is useful to estimate drug ability to decrease capillary leak stimulated by phlogistic agents. Inflammatory mediators namely prostaglandins and leukotrienes are induced by phlogistic agents. This results in arteriolar and venular dilatation and ultimately blood vessel leakage which is seen in the form of edema. Blood vessel leakage is stimulated by injection into the skin of mast cell granule release inducing substance 48/80. Leakage is characterized by penetration of dermally injected sites with Evan's blue dye.

Procedure:

Albino wistar rats weighing between 160 and 200g are used with ten rats in each group with shaving of the front of the animal. 5ml/kg of 1% Evans blue dye is injected iv. 1h later, animals are administered test drug through mouth or iv or with vehicle. After 30 minutes, the animals are administered anaesthetic agent and 0.05ml of 0.01% substance 48/80 is injected intradermally at 3 locations at left side as well as in the front. Animals are sacrificed 30 minutes later by anaesthesia. The abdominal skin is taken off and the dye penetrated cutaneous areas are measured.

Assessment: Diameter of areas showing dye penetration is measured in mm in two directions at right angles to each other and averages of injected sites in a single rat are evaluated. Percent reduction in treated rats versus controls is calculated.

b. UV light induced redness in guinea pigs:

The test was developed initially by Wilhelmi and later by Winder et al. Pre - treatment with drugs having anti-inflammatory potential delays the presence of UV light induced redness on guinea pig dermis.

Procedure:

White guinea pigs of both genders weighing about 350g are used. There are 4 guinea pigs in each group. Backs of animal are shaved 8 hours prior to test followed by chemical depilation. 24 hours later, the drug is added to solvent. Fifty percent dose is administered by gavage thirty minutes prior to UV contact. Controls are administered only vehicle. Animals are UV irradiated by keeping them in a leather covering having an opening so that they are irradiated only in the prefixed area. UV source is kept at a height of 20cm from the animal. 50% of test drug is given two

minutes after receipt of irradiation. Redness scoring is done two and four hours after irradiation.

Assessment: Severity of redness is scored by observation by two observers. Scores are put as Zero = No redness to Four = Very severe redness.

c. Croton- oil induced ear swelling:

It has 12-o-tetracanoilphorbol-13-acetate (TPA) as well as additional irritants. TPA has the ability to stimulate inflammatory mediators causing swelling.

Procedure: Ten animals are used in each group. Composition of irritant differs in concentration slightly for rats and mice. Drugs are dissolved in irritant. Irritant is applied under anaesthesia. On either parts of right ear of each mouse or rat 0.01ml or 0.02ml respectively are applied. Controls are administered vehicle alone. 4 h following irritant exposure, both ears are excised and 8mm diameter discs are punched. Weight discrepancy of two plugs is the amount of edema.

Assessment: Ability to reduce ear swelling is expressed as increment in weight of treated versus untreated ear.

d. Paw swelling in rats:

This method tests the potential of drugs to prevent edema production induced by a phlogistic agent in rat hindlimb. Many irritants like brewer's yeast, carrageenan, egg albumin, formaldehyde, dextran, kaolin, etc. have been used.

Procedure: Albino rats of either sex weighing 100 to 150 g are utilized after subjecting them to overnight fasting. The next day, rats are challenged by injecting 0.05 ml of 1% carrageenan under the skin of sole of one hind limb. A mark is made at

the lateral malleolus with ink and paw is dipped into a plethysmometer up till marked area. Paw volume is observed just following injection, at 3h, 6h and ultimately 24h after challenge.

Assessment: Increment in paw volume after 3or 6h is evaluated as % as opposed to paw measurement just after injecting carrageenan. Mean differences in test and control groups is evaluated at each time point and subjected to statistical evaluation.

e. Pleurisy test:

Irritants used to induce pleurisy in animals are histamine, bradykinin, dextran, prostaglandins, turpentine and carrageenan.

Procedure: Albino rats of 220-260g are anaesthetized and placed on their dorsum. The fur over the ribs is shaved off. Incision is made between the 7^{th} and 8^{th} rib. 0.1 ml of 1% carrageenan is introduced into pleural space. Drugs are administered orally or subcutaneously one hour before and 24h and 48h after carrageenan injection. Controls are given vehicle alone. 72h after carrageenan injection, rats are euthanized by an anaesthestic agent. 1ml of Hank's solution containing heparin is put into pleural space. Fluid is aspirated with a pipette.

Assessment: 1ml of injected Hank's solution is deducted from observed quantity of fluid to measure pleural exudate. Other parameters such as white blood cell number, lysosomal enzyme activities, fibronectin and PGE_2 levels can be measured in the exudate.

iii. **Models for determining chronic inflammation:**

Chronic (proliferative) stage of inflammation is characterized by tissue degeneration and fibrosis. The stage can be evaluated by inducing granuloma development. The following are the pharmacological methods for testing chronic inflammation:

a. Cotton wool granuloma:

Subcutaneous implantation of compressed cotton pellets induces the formation of foreign body granulomas in rats. Histopathological changes observed many days later are giant cells and connective tissue lacking differentiation apart from fluid infiltration. Connective tissue which is newly formed is evaluated by measuring weights of dried pellets after they have been removed.

Procedure: Male rats of approximately 200g are used. After anaesthesia the skin of back is removed. Lumbar region is incised following which tunnels are created just below the skin. Pellets of cotton wool are placed in the scapular region on both sides. Weight of each cotton pellet is 20mg. Test drugs are administered orally or subcutaneously for 7 days. Pellets are extracted and desiccated at sixty degree celsius for 18h and net dry weight is measured.

Assessment: Average weight of pellets of controls and treated groups are found out. Percentage decrease in granuloma weight as compared to controls is calculated.

b. Glass Rod induced granulation tissue:

Glass rod induced granulation tissue formation is a reflection of chronic proliferative inflammatory process. Wet and dry weight of connective tissue in the granuloma can be calculated apart from its chemical evaluation.

Procedure: Glass rods of 6 mm diameter and 40 mm long are used. Rods are disinfected prior to insertion. Albino rats of 130 g are administered anaesthesia and the skin over back is removed. A tunnel immediately below the skin is made and glass rod is inserted into it. Wound is closed and rods remain in place for twenty or forty days. Drugs are administered throughout the study. Animals are euthanized using anaesthetic agent. The connective tissue grows around glass rod which is removed by incising. Granuloma sac after inversion forms a plain chunk of pure connective tissue.

Evaluation: Wet weight of the granuloma tissue is recorded. Granuloma is dessicated and dry weight is noted.

c. **CFA arthritis:**

Arthritis caused by Complete Freund's Adjuvant is interpreted as an animal model of chronic inflammation. There are various widespread changes apart from affliction of synovium. These systemic manifestations are the result of penetration by white blood cells, raised white blood cells and release of reactive oxygen species. CFA consists of Mycobacterium butyricum / tuberculosis suspended in heavy paraffin oil.

Procedure: Male rats of 130 -200g are chosen. 0.1ml of CFA is injected subcutaneously into the footpad of rat. Drugs are administered upto day 12. Paw volume is measured on days 0, 3, 7, 14 and 21 using a plethysmometer.

Assessment: Reduction in paw edema stimulated by is taken as index of inflammation reducing ability of test drug. Other supplementary parameters include measurement of oxidative stress markers, cytokines with pro-inflammatory potential and histopathological studies to establish the possible means underlying the inflammation reducing potential of test drugs.

1.3 JUSTIFICATION

- As per the literature reviewed the studies done so far have studied the effects of ketogenic diet and intermittent fasting on various animal models of inflammation.

- To date there have been no studies which have tested the long term effects of various diets and intermittent fasting on the animal model used in the present study (CFA induced inflammation).

- A single study which has compared the effects of various diets and IF on inflammation with various interventional groups running in parallel has not been done so far.

- No studies done so far, have tested the effect of a combination of intermittent fasting with ketogenic diet.

1.4 OBJECTIVES:

Primary objective:

- To determine the effects of ketogenic diet, intermittent fasting, high carbohydrate diet and a combination of intermittent fasting with ketogenic diet on chronic inflammation induced by Complete Freund's Adjuvant (CFA) in male Wistar rats.

Secondary objective:

- To determine the effects of ketogenic diet, intermittent fasting, high carbohydrate diet and a combination of intermittent fasting with ketogenic diet on oxidative stress parameters in male Wistar rats.

2. MATERIALS AND METHODS

2.1 Materials:

a. Complete Freund's Adjuvant was bought from Sigma Aldrich.

b. Digital plethysmometer was sold by Orchid Scientifics, Nashik.

c. Ketone monitoring meter (Precision Xtra Ketone Monitoring System) and strips were purchased from Abbott.

d. Kit for estimating TBARS was sold by Bioassay Systems, United States of America.

e. Enzyme Linked Immunosorbent Assay (ELISA) kits for measuring antioxidant enzymes were obtained from MyBioSource, Inc, California.

f. Enzyme Linked Immunosorbent Assay kits meant to estimate cytokines were sold by Krishgen Biosystems, Mumbai.

g. Dietary ingredients were brought from local shops except protein powder which was bought from Isopure sold on Amazon.

2.2 METHODS

2.2.1 Animals procurement:

Male Wistar rats weighing 150-200g were procured from Institutional Animal House. The study was approved by IAEC (Letter no.7/A dated 18/05/2016). Study was done in accordance with guidelines put up by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Animal grouping is as described in Table 1.

Table 1: Grouping of Animals:

Groups	**Type of Diet**
Group I (Control group)	Standard diet
Group II	Ketogenic Diet (KD)
Group III	Intermittent fasting (IF) (Fasting and feeding on alternate days) Standard diet fed on feeding days
Group IV	Intermittent fasting + Ketogenic Diet (IF+KD) (Fasting and feeding on alternate days) Ketogenic diet fed on feeding days
Group V	High carbohydrate diet (HCD)
Group VI – Normal group	Standard diet
All groups were fed the respective diets as mentioned above four weeks prior to injection of CFA and respective diets were fed all along the study. Food was given on at libitum basis. Diets were made based on previous similar studies and details are elaborated in Table 2.[12, 88]	

Table 2: Composition of various diets:

Ingredients g/ 100g	Standard diet	Ketogenic diet	High Carbohydrate diet
Ghee	05	60	05
Coconut Oil	-	09	-
Whey protein	25	20	26
Wheat flour	50	01	
Maida (White Flour)	-	-	32
Cane Sugar	-	-	32
Bran	20	10	05
Total	100	100	100

2.2.2 Inflammatory stimulus injection:

0.1ml of CFA in a 1mg/ml concentration which was the inflammatory stimulus was injected below the skin at the plantar aspect of right hind paw. All animals received the inflammatory stimulus except those in Group VI (Normal group).

2.2.3 Measurement of blood ketone levels:

Blood ketone levels were measured before starting the respective diets and at the end of the study by tail vein puncture using ketone monitoring strips and meter.

2.2.4 Assessment of Inflammation and Oxidative stress:

Animals were anaesthetized by thiopentone injected intraperitoneally after 21 days of injection of CFA and blood obtained by cardiac puncture to estimate TNF α, IL-1β, TBARS, SOD and catalase. After blood collection animals were euthanized by an overdose of thiopentone administrated by intraperitoneal route. The hind limbs were amputated after euthanizing the animals.[89]

i. Estimation of inflammatory mediators:

A mark was made on the right hind limb at the malleolus with indelible ink to ensure uniform dipping at subsequent readings. Volume of right hindlimb was estimated on days 0,3,7,14 and 21 by digital plethysmometer.[87] Percentage inhibition of edema was calculated using the following formula:[86]

$$\text{Percentage inhibition of edema} = 1 - \frac{\text{(Mean increase in paw volume in treated group)}}{\text{(Mean increase in paw volume in control group)}} \times 100$$

TNF α, IL-1β and CRP in serum were measured by ELISA kits as per manufacturer's directions. The ankle joints of 5 animals from each group were subjected to histopathological analysis of inflammation.

ii. Measurement of antioxidant enzymes:

Superoxide dismutase and catalase in blood were measured by ELISA kits. Thiobarbituric acid reducing substance (TBARS) was estimated colorimetrically. All these were estimated according to the procedure given by the manufacturer. Paw tissues of 5 animals from each group were dissected out and crushed for analysis of oxidative stress parameters mentioned above.

3. DATA ANALYSIS PLAN

Statistical software used was Graph Pad Prism. Data was represented as Mean ± Standard Deviation (SD). Differences between groups were evaluated by One Way Analysis of Variance (ANOVA) after which Post Hoc Dunnett's test was carried out. P value of < 0.05 was considered significant.

4. RESULTS

Current study was carried out to analyze the roles of ketogenic diet, intermittent fasting, ketogenic diet combined with intermittent fasting and high carbohydrate diet in Complete Freund's Adjuvant stimulated inflammation in rats.

4.1 Effect of various diets on weight of rats:

Mean ± SD (g)	Control	KD	IF	IF + KD	HCD	Normal	F value	P value
Baseline weight	166.2 ± 13.4	169.9 ± 8.3	168.3 ± 11.6	163.3 ± 9	162.2 ± 10	164.4 ± 13.6	0.7045	0.6225
Final weight	221.5 ± 43.7	191.5 ± 6.7*	185.3 ± 12.4**	156.7 ± 17.2 ****	230± 30.9	239.2 ± 14	16.72	< 0.0001

Table 3: Effect of various diets on weight of rats. KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

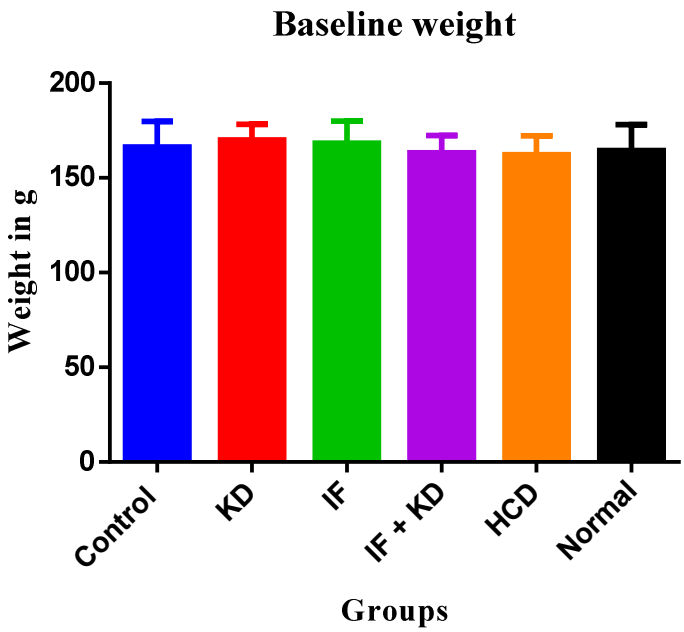

Figure 1: Baseline weight of rats: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

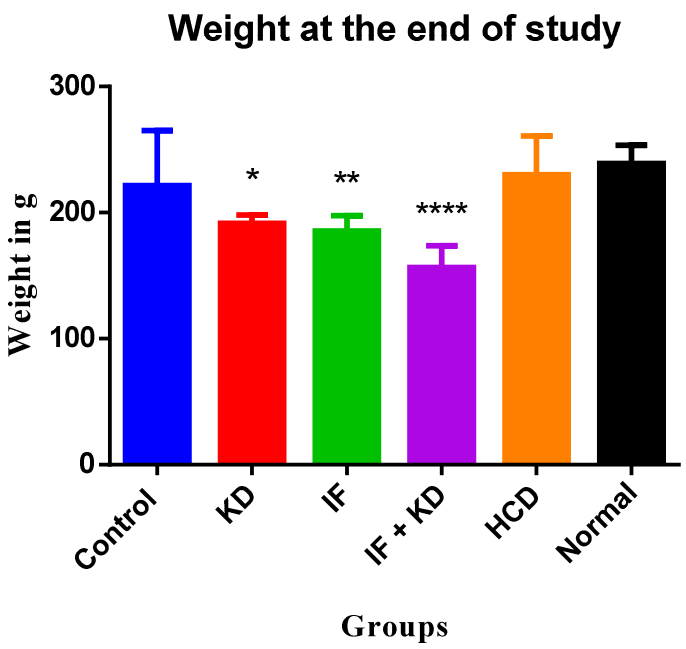

Figure 2: Effect of various diets on weight of rats at the end of study: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$

Current study found that, there was a significantly less increase in weight at the end of the study in the various interventional (KD, IF and IF + KD) groups as opposed to control group. HCD and normal groups showed no major difference in weight as opposed to control group (Table 3, Figures 1 and 2).

4.2: Effect of various diets on blood ketones:

Mean ± SD (mg/dl)	**Control**	**KD**	**IF**	**IF + KD**	**HCD**	**Normal**	**F value**	**P value**
Baseline ketone levels	0.72 ± 0.12	0.63± 0.14	0.58 ± 0.12	0.61± 0.13	0.61 ± 0.17	0.64 ± 0.17	1.038	0.405
Final ketone levels	0.72 ± 0.13	2.23 ± 0.22 ****	2.51 ± 0.27 ****	2.74 ± 0.59 ****	0.67 ± 0.27	0.71± 0.2	97.32	< 0.0001

Table 4: Effect of various diets on blood ketone levels: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

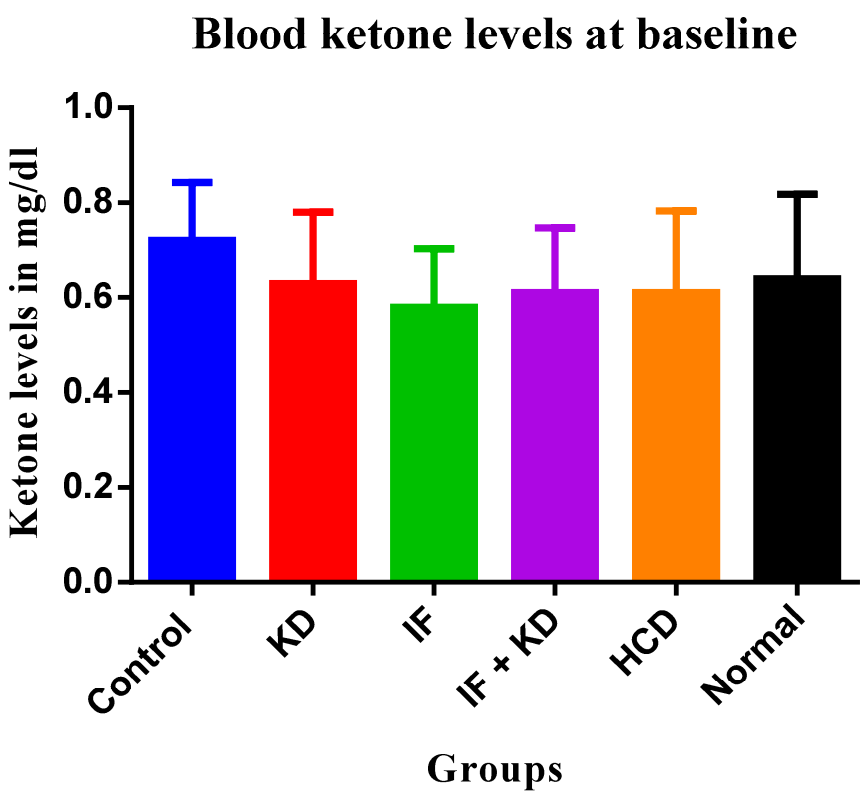

Figure 3: Effect of various diets on blood ketone levels at baseline: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

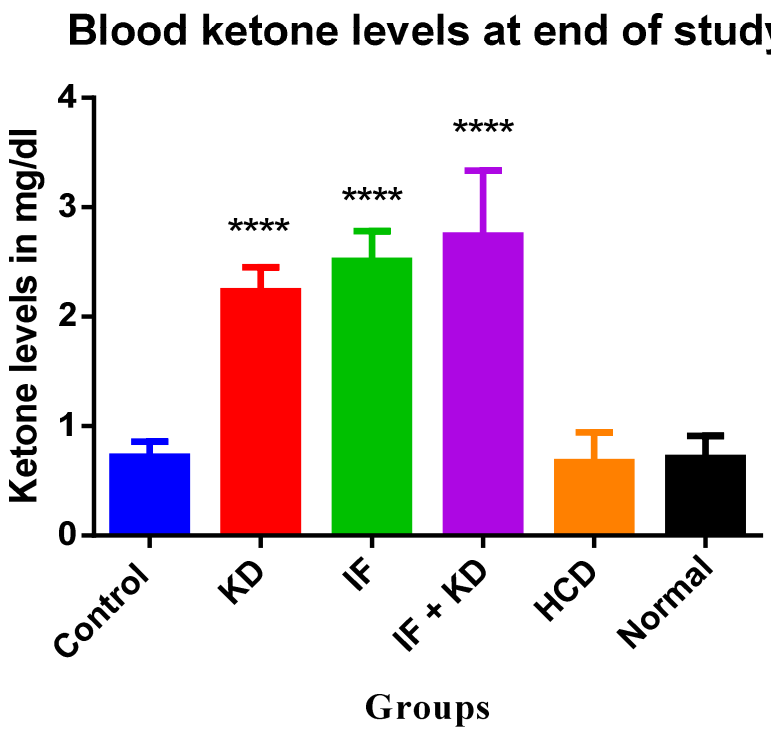

Figure 4: Effect of various diets on blood ketone levels at the end of study: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

Current study found that, there was a significant rise in blood ketone levels at the end of the study in the various interventional (KD, IF and IF + KD) groups as opposed to control group. HCD and normal groups showed no major difference in ketone levels as opposed to control group at the end of the study (Table 4, Figures 3 and 4).

4.3: Effect of various diets on CFA induced paw edema:

Mean ±SD (ml)	Control	KD	IF	IF + KD	HCD	Normal	F value	P value
Day 0	1.071 ± 0.118	1.025± 0.095	0.983± 0.088	0.975± 0.059	1.08± 0.134	1.057± 0.109	1.885	0.1122
Day 3	1.729 ± 0.132	1.432 ± 0.118 **** (17.2%)	1.516 ± 0.07 *** (12.4%)	1.419 ± 0.076 **** (18%)	2.162 ± 0.137 ****	1.124 ± 0.085 **** (35%)	108.6	< 0.0001
Day 7	1.518 ± 0.135	1.263 ± 0.104** (16.8%)	1.342 ± 0.11* (11.6%)	1.234 ± 0.186*** (18.8%)	1.696 ± 0.213*	1.160 ± 0.077**** (23.6%)	19.05	< 0.0001
Day 14	1.793± 0.121	1.378± 0.086 **** (23.2%)	1.443± 0.08 **** (19.6%)	1.264± 0.149 **** (29.6%)	2.2± 0.188 ****	1.032± 0.086 **** (42.5%)	110.8	< 0.0001
Day 21	2.095 ± 0.121	1.162 ± 0.106 **** (44.6%)	1.323 ± 0.132 **** (36.9%)	1.086 ± 0.181 **** (48.2%)	2.27 ± 0.169 *	1.185 ± 0.065 **** (43.5%)	149.3	< 0.0001

Table 5: Effect of various diets on CFA induced paw edema: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$. Values in brackets indicate percentage inhibition

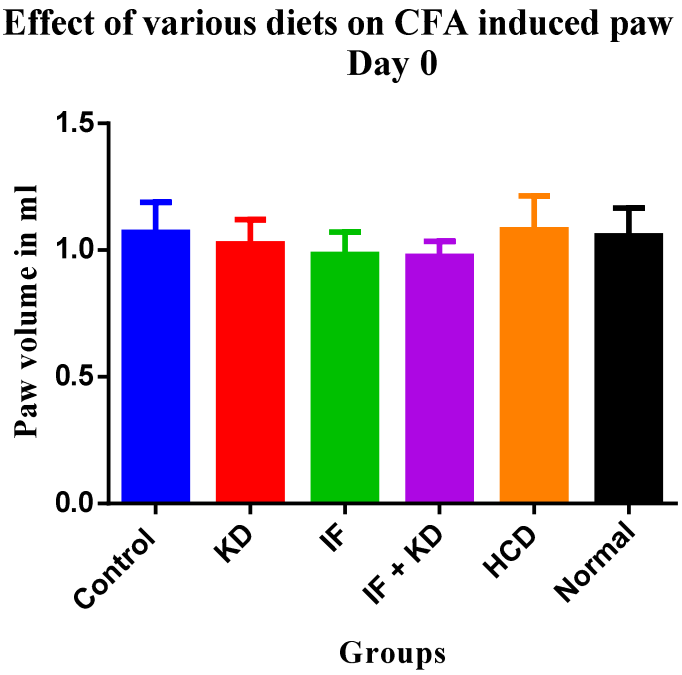

Figure 5: Effect of various diets on CFA induced paw edema (Day 0): KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

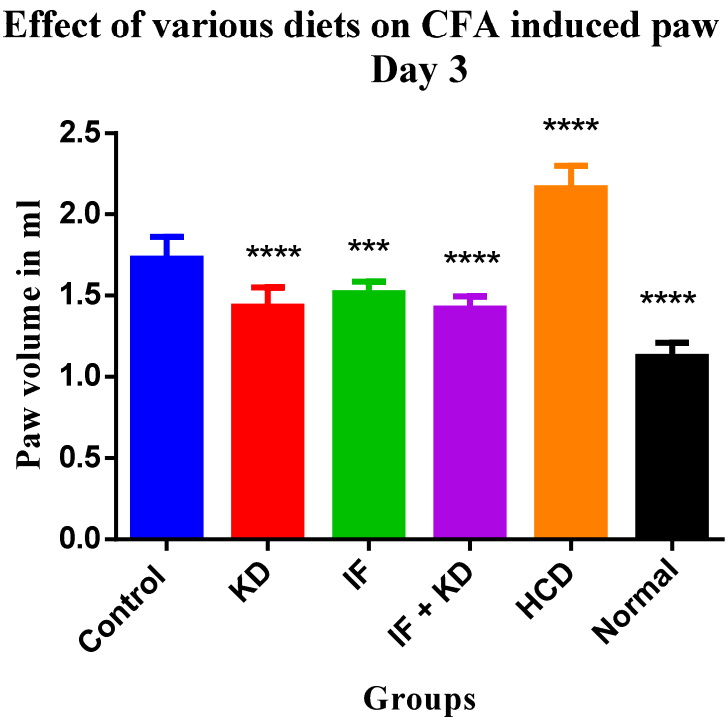

Figure 6: Effect of various diets on CFA induced paw edema (Day 3): KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

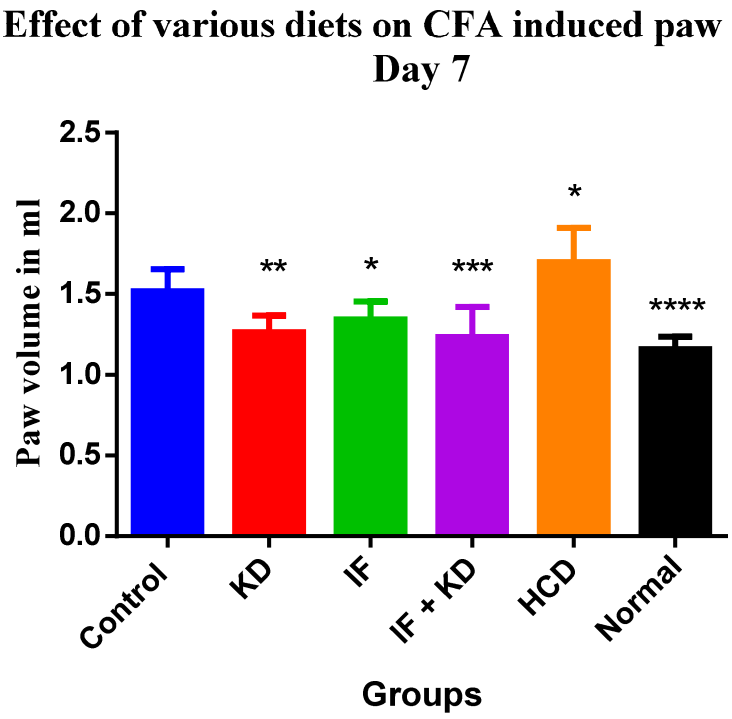

Figure 7: Effect of various diets on CFA induced paw edema (Day 7): KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

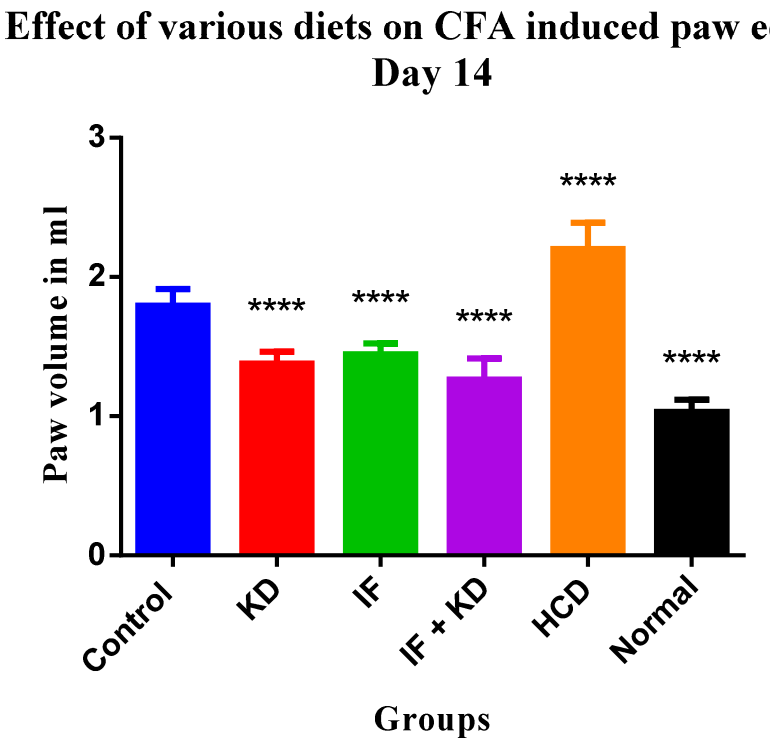

Figure 8: Effect of various diets on CFA induced paw edema (Day 14): KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

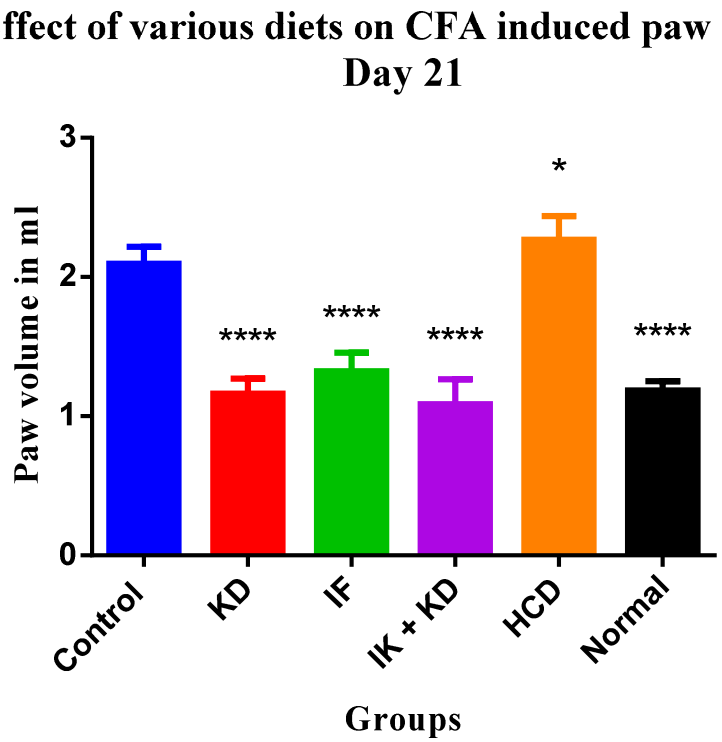

Figure 9: Effect of various diets on CFA induced paw edema (Day 21): KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

Current study found that, there was significantly less rise in paw edema in the various interventional (KD, IF and IF + KD) groups as opposed to control group. In the HCD group there was a significant rise in paw edema as compared to control group (Table 5, Figures 5 to 9).

4.4: Effect of various diets on inflammatory cytokines and CRP

Mean ±SD	Control	KD	IF	IF + KD	HCD	Normal	F value	P value
Serum IL-1β (pg/ml)	652.5 ± 30.79	167.9 ± 7.92 ****	256.4 ± 26.21 ****	141.6 ± 8.24 ****	945.1 ± 34.45 ****	113.7 ± 8.3 ****	2310	< 0.0001
Serum TNF - α (pg/ml)	37.54 ± 9.37	23.08 ± 4.21 ****	21.51± 4.32 ****	23.12 ± 3.97 ****	48.75 ± 7.82 ***	15.71 ± 2.08 ****	44.47	< 0.0001
Serum CRP (mcg/ml)	869 ± 26.3	509.2 ± 38.3 ****	581.5 ± 50.6 ****	383.5 ± 44.4 ****	941 ± 27.9 ***	283.2 ± 8.6 ****	545.8	< 0.0001

Table 6: Effect of various diets on inflammatory cytokines and CRP: CRP: C reactive protein, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

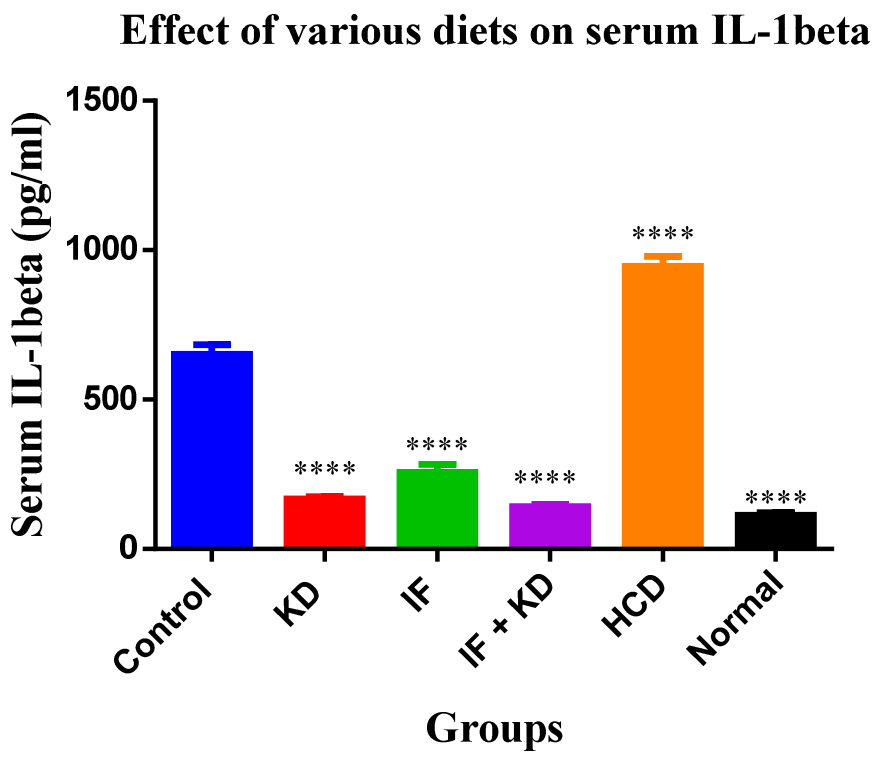

Figure 10: Effect of various diets on serum IL-1beta: IL-1 beta: Interleukin-1 beta**,** KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

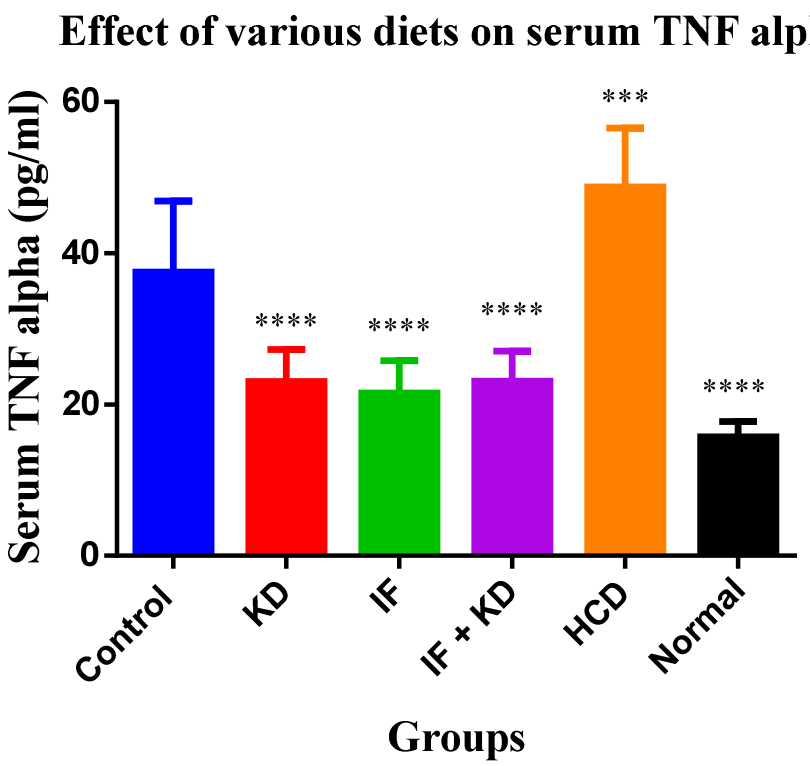

Figure 11: Effect of various diets on serum TNF alpha: TNF – alpha – Tumor necrosis factor alpha**,** KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

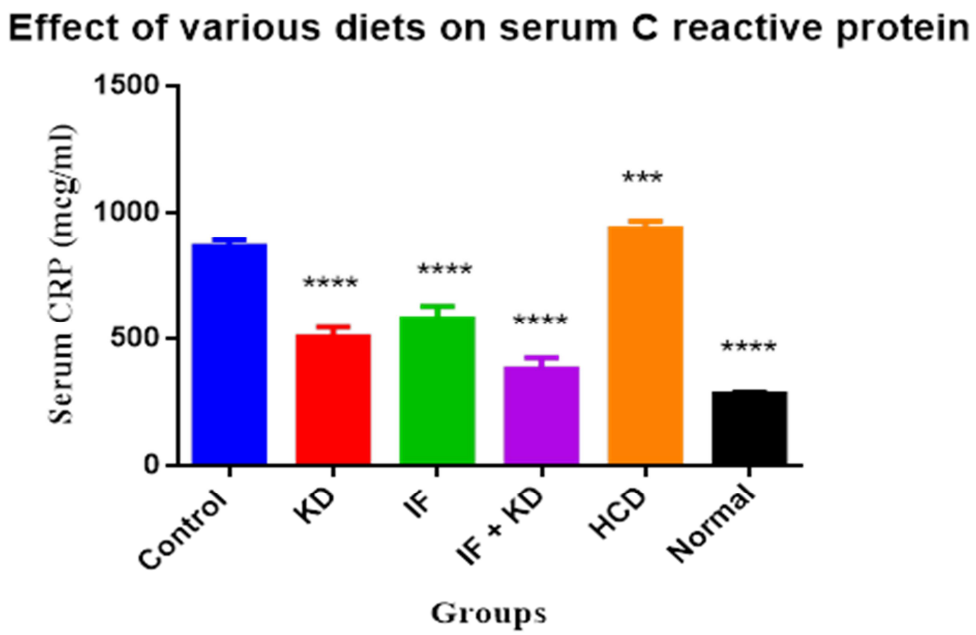

Figure 12: Effect of various diets on serum CRP: CRP – C reactive protein**,** KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$

Current study found that, there was a significantly less rise of tumor necrosis factor alpha, interleukin -1 beta and CRP of various interventional (KD, IF and IF + KD) groups as opposed to the control group. In HCD group there was a significant rise of tumor necrosis factor alpha, interleukin -1 beta and CRP as opposed to the control group (Table 6, Figures 10, 11 and 12).

4.5: Effect of various diets on oxidative stress parameters in serum and hind paw tissue:

Mean ±SD	Control	KD	IF	IF + KD	HCD	Normal	F value	P value
Serum catalase ng/ml	60.87 ± 7.11	118.9 ± 10.32 ****	164.3 ± 9.36 ****	122.2 ± 12.77 ****	25.35 ± 12.13 ****	244.3 ± 6.71 ****	596.7	< 0.0001
Serum SOD U/ml	140± 4.22	182.4 ± 15.15***	183.1± 11.93 ****	186.1± 9.85****	91.49± 4.33 ****	231.7± 44.47 ****	55.34	< 0.0001
Serum TBARS mMol	14.79 ± 2.87	8.2± 3.36 ****	10.99± 2.76 **	10.12± 2.08 ***	18.11± 0.8*	4.48± 1.47 ****	40.46	< 0.0001

Table 7: Effect of various diets on oxidative stress parameters in serum: SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reducing substance, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

Mean ±SD	Control	KD	IF	IF + KD	HCD	Normal	F value	P value
Tissue catalase ng/ml	117.2 ± 5.58	235.2 ± 13.55 ****	208.4 ± 3.78 ****	232.3 ± 25.44 ****	69.52 ± 20.26 **	367 ± 33.01 ****	136.3	< 0.0001
Tissue SOD U/ml	112.9 ± 4.01	144.9 ± 4.31 ****	134.2 ± 4.47 ****	156.1± 2.32 ****	92.22 ± 2.32 ****	178.3 ± 6.26 ****	270.5	< 0.0001
Tissue TBARS mMol	18.14 ± 1.79	12.62 ± 0.5 ****	13.78 ± 0.69 ****	10.8 ± 0.78 ****	15.68 ± 0.34 **	3.58 ± 0.75 ****	143.4	< 0.0001

Table 8: Effect of various diets on oxidative stress parameters in tissues: SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reducing substance, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data was analyzed by ANOVA followed by Post Hoc Dunnett's test. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

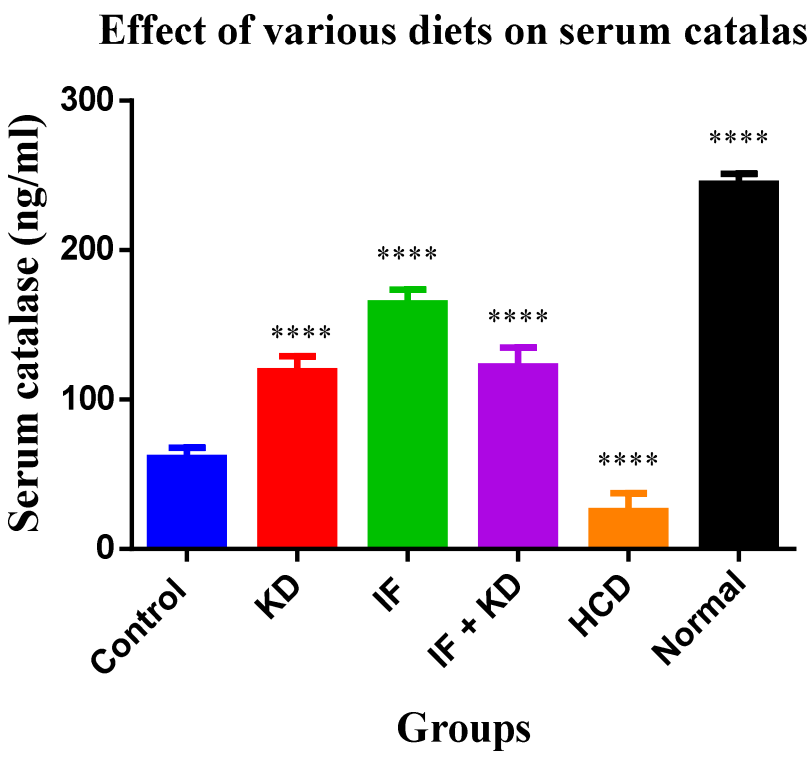

Figure 13: Effect of various diets on serum catalase: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

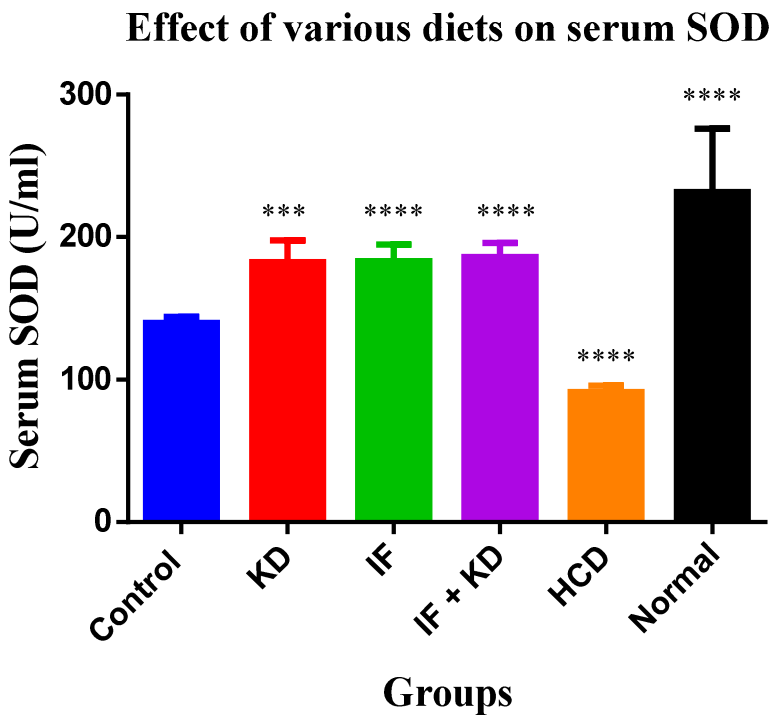

Figure 14: Effect of various diets on serum SOD: SOD: Superoxide dismutase, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p< 0.0001$

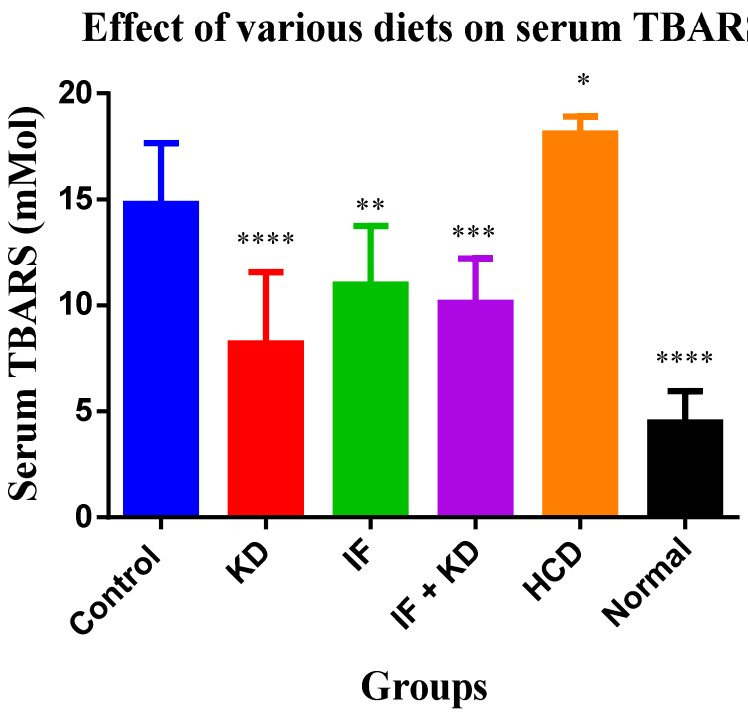

Figure 15: Effect of various diets on serum TBARS: TBARS: Thiobarbituric acid reducing substance, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

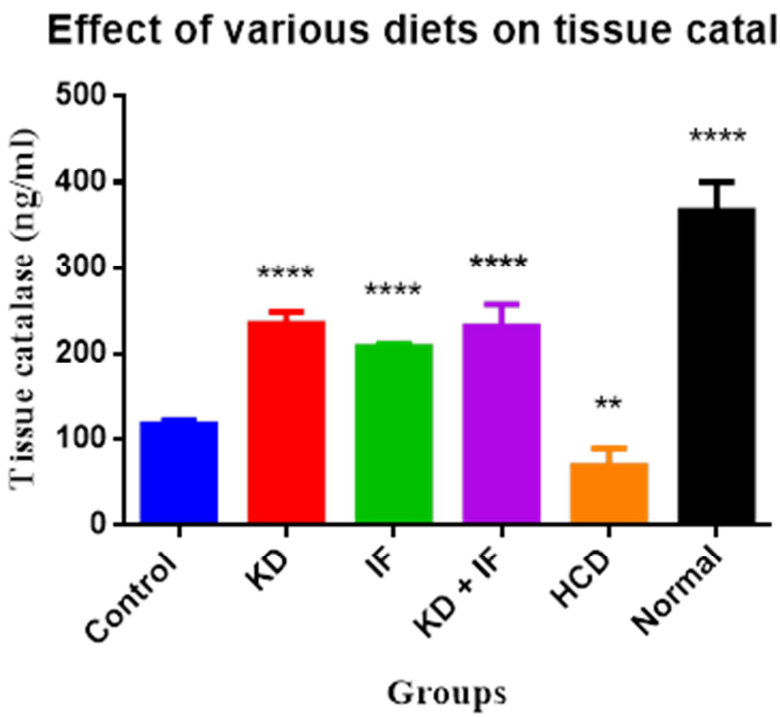

Figure 16: Effect of various diets on tissue catalase: KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

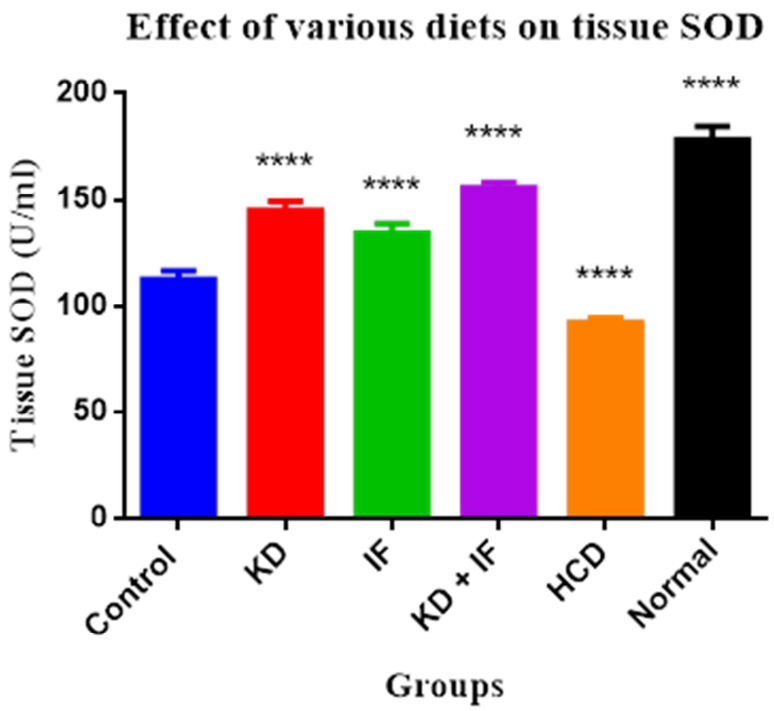

Figure 17: Effect of various diets on tissue SOD: SOD: Superoxide dismutase, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

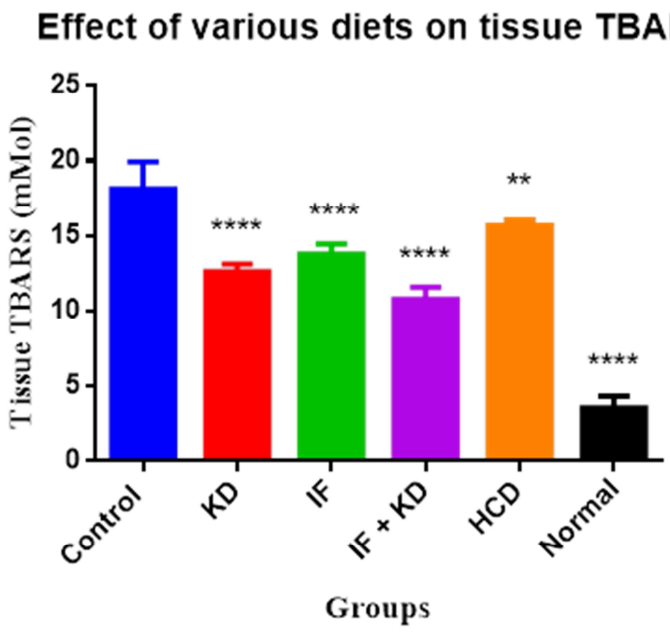

Figure 18: Effect of various diets on tissue TBARS: TBARS: Thiobarbituric acid reducing substance, KD: Ketogenic diet, IF: Intermittent fasting, IF + KD – Intermittent fasting + Ketogenic diet, HCD – High carbohydrate diet. Data evaluated by ANOVA after which Post Hoc Dunnett's test was carried out. $P < 0.05$ considered as significant. Asterisk indicates that Post Hoc Dunnett's test p value is significant. *p < 0.05, **p < 0.01, *** p < 0.001, ****p< 0.0001

Current study found that, there was a significantly less depletion of superoxide dismutase and catalase in serum and hind paw tissue in the various interventional (KD, IF and IF + KD) groups as opposed to control group. Also, there was a significantly less rise in TBARS in KD, IF and (IF + KD) groups as opposed to control group in serum and hind paw tissue. The HCD group showed higher depletion of superoxide dismutase and catalase in serum and hind paw tissue as opposed to control group. Also, there was a significant rise in TBARS in HCD group in hind paw tissue (Tables 7 and 8, Figures 13 to 18).

4.6: Histopathological examination of the ankle joint:

The ankle joints (injected with CFA) of animals belonging to various experimental groups were subjected to histopathological examination after staining with haematoxylin and eosin. The bone and cartilage of the joint was examined for the presence of inflammatory infiltrate in the form of neutrophils and lymphocytes. The histopathology has been depicted in the form of photomicrographs in figures 19 to 25.

Figure 19: Photomicrograph of normal ankle joint

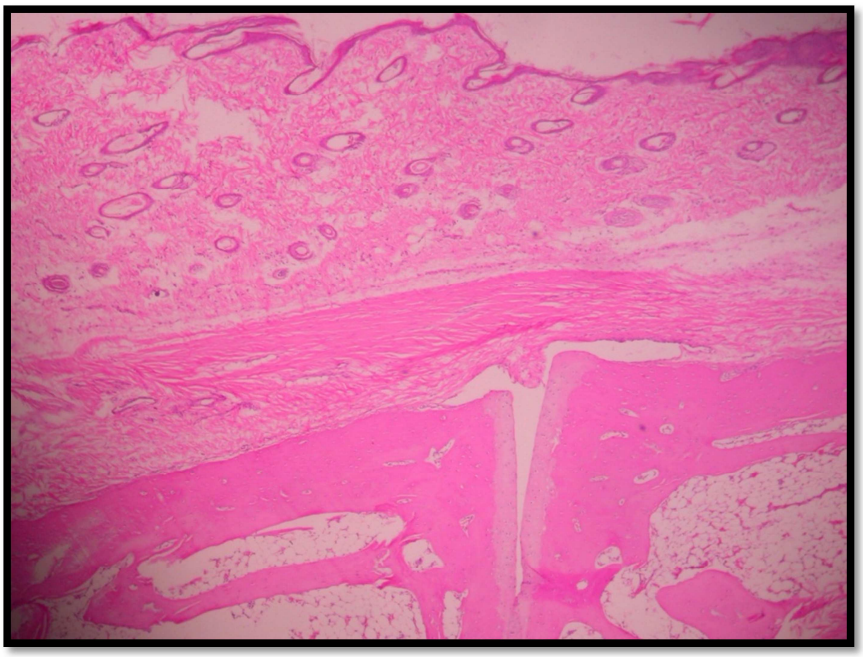

Figure 19 shows a normal ankle joint of Wistar rat with normal cartilage and bone

Figure 20: Photomicrograph of normal ankle joint

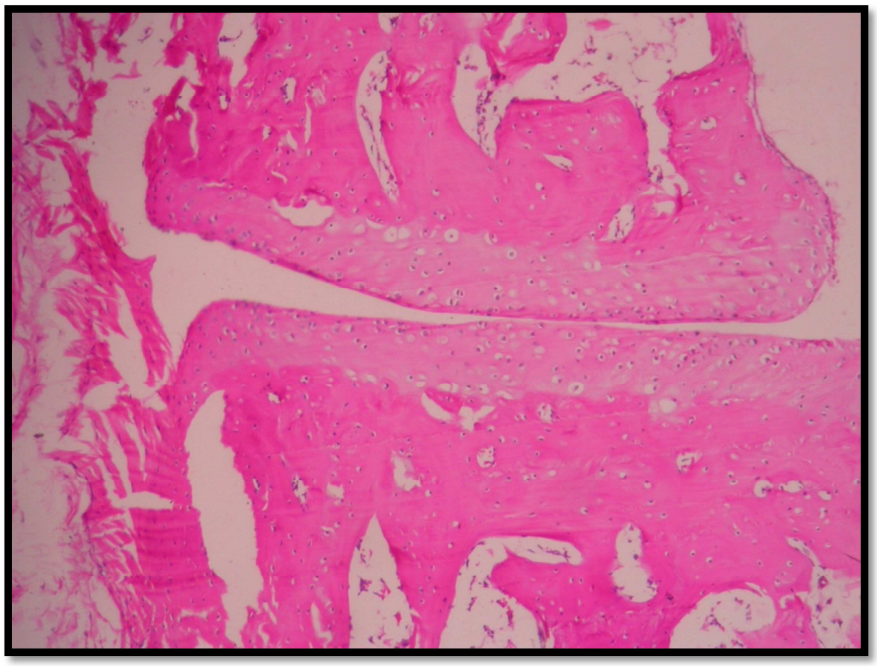

Figure 20 shows a normal ankle joint of Wistar rat with normal cartilage and bone

Figure 21: Photomicrograph of ankle joint of control group

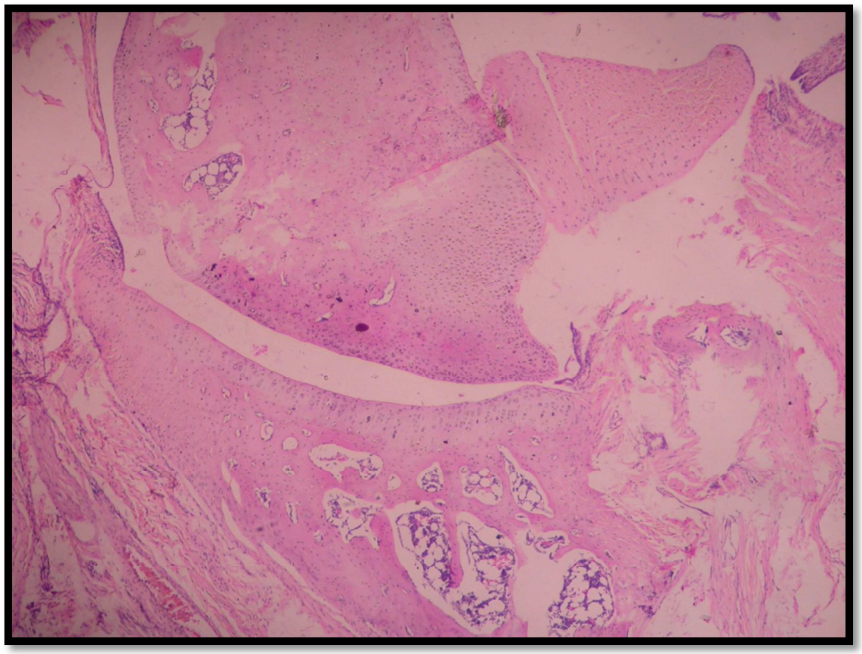

Figure 21: Ankle joint of Wistar rat of control group showing vast inflammatory infiltrate

Figure 22: Photomicrograph of ankle joint of KD group

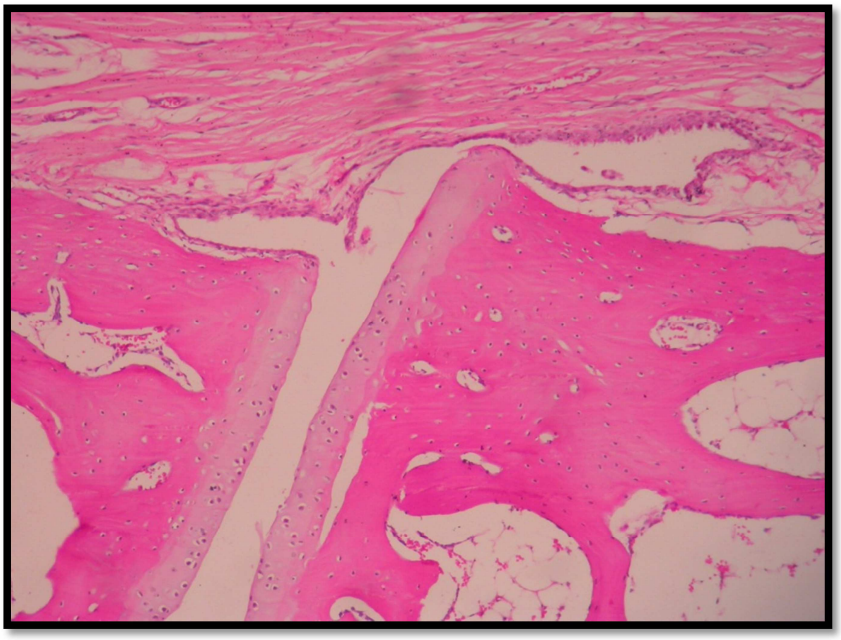

Figure 22: Ankle joint of Wistar rat of KD group showing scanty inflammatory infiltrate; KD group – Ketogenic diet group

Figure 23: Photomicrograph of ankle joint of IF + KD group

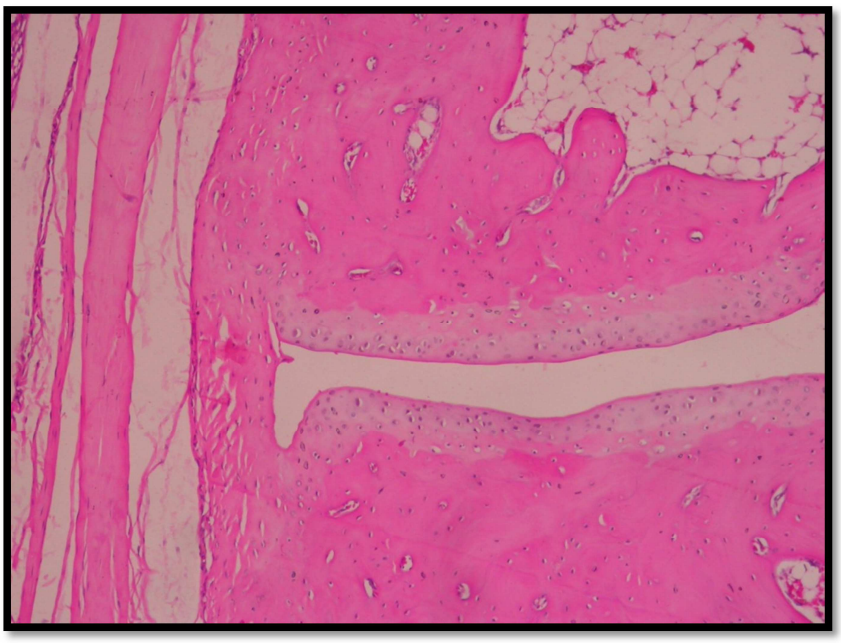

Figure 23: Ankle joint of Wistar rat of (IF + KD) group showing scanty inflammatory infiltrate; (IF + KD) group – (Intermittent fasting + Ketogenic diet) group

Figure 24: Photomicrograph of ankle joint of IF group

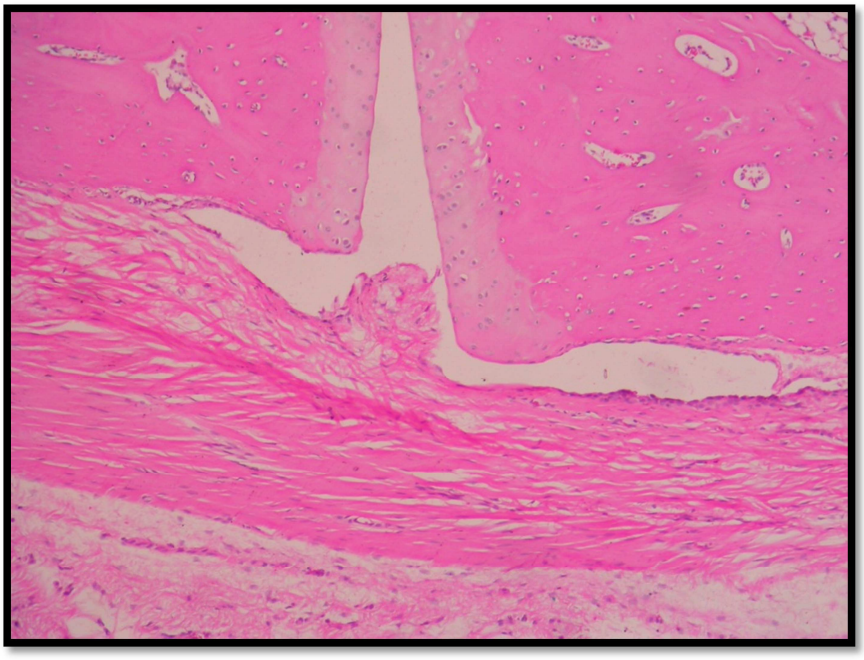

Figure 24: Ankle joint of Wistar rat of IF group showing scanty inflammatory infiltrate; IF group – Intermittent fasting group

Figure 25: Photomicrograph of ankle joint of HCD group

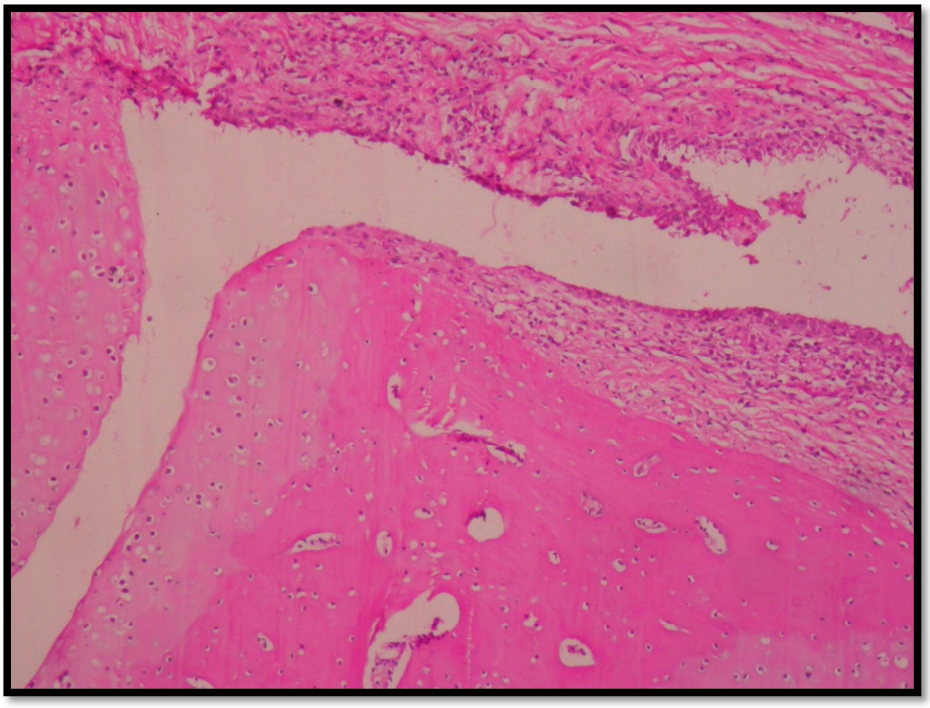

Figure 25: Ankle joint of Wistar rat of HCD group showing vast inflammatory infiltrate; HCD group – High carbohydrate diet group

5. DISCUSSION

Current study investigated the part played by ketogenic diet, intermittent fasting, a combination of ketogenic diet and intermittent fasting as well as high carbohydrate diet in the inflammatory process stimulated by CFA in male Wistar rats. It was found that, KD and IF alone and in combination can curb the inflammatory process by reducing paw edema, cytokines of pro-inflammatory nature, CRP and vulnerability to oxidative stress and inhibiting exhaustion of antioxidant enzymes. As opposed to this current study also found that, carbohydrate rich food fed for seven weeks worsens inflammatory process triggered off by CFA. With the carbohydrate rich diet, inflammatory process persists for 21days. Further, there is an increase in cytokines favouring inflammation, increased vulnerability to oxidative stress, CRP and significant exhaustion of antioxidant enzymes.

The current study used CFA as a pro-inflammatory agent because it induces prolonged inflammation and hence effect of various diets on inflammation can be studied over a long period.

Most crucial components of carbohydrate rich diet were sucrose (a combination of glucose and fructose) and maida (white flour) which are used in large quantities in manufacturing junk food which contributes majorly to pathogenesis of non -communicable diseases such as diabetes, obesity and coronary artery disease.

Studies done previously have studied parts played by ketogenic diet and intermittent fasting in inflammatory processes over a short duration of twenty four to forty eight hours.[10,11,12,13] The current study has studied effects of various dietary interventions over twenty one days. Study has probed into not just their effects on

paw edema visible to the naked eye, but also on how histopathology and markers such as pro- inflammatory cytokines, CRP and antioxidant enzymes are influenced.

Ketosis can be accomplished by caloric restriction or by cutting down on carbohydrates through a ketogenic diet.[90] This is a physiological phenomenon in contrast to ketoacidosis a consequence of uncontrolled diabetes, where in ketones measure 20mmol/l of blood with concomitant reduction of blood pH. In ketosis by caloric restriction or ketogenic diet, ketones rise to 7/8 mmol/l of blood with no pH change.[18]

Ketogenic diets have a high proportion of fat, proteins in moderate amounts and low-carbohydrate content causing enhanced fat burning and restricted breakdown of carbohydrates and proteins. A state of metabolism called physiological ketosis results in which increased fat-derived ketones and decreased glucose are found in the blood. Here body depends on calories from ketolysis in comparison to glycolysis, where glucose is the chief source of energy.[90]

Various means of fasting have been tried and tested by animal and human experiments. Commonly used regimens are food deprivation on alternate days with food consumption on non-fasting days and 5:2 diet where in no food is consumed on two non-successive days of the week and person eats for the rest of the week. Time bound eating is one in which the person eats only within a fixed time frame everyday day causing long fasting periods.[19,91]

Current study measured rat paw edema by a digital plethysmometer which found a decrease in edema in all dietary interventions compared to control group during the prefixed times when edema was measured except in high carbohydrate diet

group where paw edema increased significantly. So far only one article published states that, CFA induced paw edema in rats is decreased by ketogenic diet. Here, paw edema has been evaluated 48 hours after CFA administration. But, the current study has measured paw edema over various periods as mentioned earlier and has shown that KD and IF alone and in combination reduce edema over twenty one days.[12]

In the present study all dietary interventions except HCD have found to reduce inflammation in the paw tissue as seen in sections of the ankle joint. Sections of the ankle joint in the CFA injected hind paw show reduction in neutrophils and lymphocytes by all dietary interventions as compared to the control group except in the HCD group. The HCD group shows widespread inflammation in the ankle joint just as in the control group.

In current study, cytokines in blood specifically TNFα and interleukin -1β of pro-inflammatory nature have been decreased by all dietary interventions except group fed with high carbohydrate where these cytokines were elevated significantly. This study adds to the evidence that decrease in these cytokines is possible by IF and KD but experiments in this field have not used the same animal model.[10,11]

Current study has found that the cytokines mentioned above were increased by the carbohydrate rich diet. Similar results were found in a study which studied role of fructose and sucrose on cytokines.[92]

Elevated CRP is an undisputable indication of ongoing inflammation. Inflammation favouring consequences of CRP are complement activation apart from recruitment of monocytes synthesizing cytokines. CRP at times can exacerbate tissue

injury.[43,44] The present study found that all dietary interventions reduce CRP in comparison to the control group, except high carbohydrate diet which increases CRP.

Current study measured oxidative stress marker namely TBARS and enzymes having antioxidant potential namely superoxide dismutase as well as catalase in blood. Different dietary interventions decreased TBARS and prevented exhaustion of superoxide dismutase as well as catalase. The exception was the high carbohydrate diet group in which TBARS was elevated and superoxide dismutase and catalase were significantly depleted as opposed to controls. Present study has found similar results in hind paw tissue of rats. TBARS is a marker of lipid peroxidation and thereby oxidative stress.[93]

A study where in a diet high in sugar was administered to rats for 14 days also found elevation of TBARS in blood and reduction in superoxide in hearts of the animals.[94]

Previous studies indicate that, ketogenic diet prevents NF-*κ*B from getting activated and by doing so prevents activation of pathways influenced by NF-*κ*B. Fasting decreases NFκB levels and hence both ketogenic diet and fasting prevent generation of interleukins and tumor necrosis factor α and suppress actions of cyclooxygenase-2 and inducible nitric oxide synthase.[95,96,97]

Diets loaded with sucrose / fructose activate NF-κB by alteration of microorganisms colonizing the gut. Enhancing levels of gram negative organisms causes release of large amounts of LPS which enhances certain receptors to which it binds lead to activation of NF-κB.[92,98]

NF-kB controls expression of various genes concerned with inflammation. Synthesis of inflammation favouring cytokines namely IL-1, IL-6 and TNF- α is under control of NF-kB. NF-kB signaling contributes to pathogenesis of diseases of metabolism namely obesity, T2DM and coronary artery disease.[99]

Both fasting and ketogenic diet activate antioxidant defences within the body. Relevant is stimulation of Nuclear Factor Erythroid-derived 2 (NF-E2)-related factor 2 (Nrf2) which controls adaptation of the body to internal and external sources of stress. Among the varied roles of Nrf2, relevant ones are directly protecting against oxidative stress, coding for antioxidant enzymes, augmenting repair as well as disposal of denatured proteins and suppressing cytokine enhanced inflammatory process.[91, 95, 100]

Among ketones, βHB, is a suppressor of Histone Deacetylases of classes I and IIa. This suppression leads to enhanced expression of antioxidant genes coding for catalase, mitochondrial superoxide dismutase and metallothione 2. Other mechanisms underlying oxidative stress nullifying property of ketogenic diet include control of NAD+/NADH ratio which protects against reactive oxygen species. Also ketogenic diet enhances capability of electron transport chain by increasing levels of uncoupling proteins (UCPs).[100]

Fasting reduces synthesis of ROS by mitochondria by enhancement of UCPs. These proteins are present on inner mitochondrial membrane and cause proton leak into the matrix. This uncouples electrochemical gradient from ATP synthesis which causes a fall in membrane potential of mitochondria and hence causes reduction in synthesis of ROS. [96, 97]

6. SUMMARY

It has been recognized from time immemorial that diet is crucial in causation and management of disease – especially in chronic Non Communicable Diseases (NCDs). NCDs have overtaken infectious diseases and pose a threat to the developed and developing world. Inflammation with its cardinal features of swelling, redness, etc may be beneficial during short periods in helping tissues heal. Diseases of metabolism such as diabetes mellitus, obesity, ischaemic heart disease and fatty liver (not caused by chronic alcoholism) are evidence to the fact that, inflammation in the long term is not helpful. Factors such as migration to cities as well as increased advertising and affordable prices of junk food are causing increased intake of refined grains, sweet beverages and bakery products which enhance the possibility of developing NCDs. Diet rich in carbohydrate is a crucial risk factor in causation of NCD and long standing inflammatory process underlies the pathogenesis of NCDs. Hence an economical and rational method to diminish the possibility of developing such diseases would involve reducing carbohydrate consumption by KD or IF. Hence, the current study was undertaken with an objective of determining roles of ketogenic diet, intermittent fasting and high carbohydrate diet in inflammatory process stimulated by CFA in male Wistar rats.

Current study found that, KD and IF can curb the inflammatory process by reducing paw edema, reducing cytokines of pro-inflammatory nature, CRP and reducing vulnerability to oxidative stress and inhibiting exhaustion of antioxidant enzymes. As opposed to this current study also found that, carbohydrate rich food fed for seven weeks worsens inflammatory process triggered off by Complete Freund's Adjuvant. With the carbohydrate rich diet, inflammatory process lasts continuously

for 21days. Further, there is an increase in cytokines favouring inflammation, CRP, increased vulnerability to oxidative stress and significant exhaustion of antioxidant enzymes.

Findings of current study have found that inflammatory process is modifiable by two dietary means both involving carbohydrate restriction since it has been found that carbohydrate is the main culprit which contributes to the pathogenesis of inflammation and in turn NCDs. Hence, the two dietary means are practicing intermittent fasting or consuming a ketogenic diet. Carbohydrate forms a substantial chunk of most food that humans especially the Indian population consumes. Therefore, restricting carbohydrate by means of a ketogenic diet may not be practicable. Hence, a more feasible alternative would be intermittent fasting alone rather than routine carbohydrate restraint by way of a ketogenic diet. Both modalities seem to have comparable potential in interfering with the progression of the inflammatory process.

7. CONCLUSION

- Findings of current study point out that inflammatory process is modifiable by two dietary means both involving carbohydrate restriction since it has been found that carbohydrate is the main culprit which contributes to the pathogenesis of inflammation and in turn NCDs. Hence, the two dietary means are practicing intermittent fasting or consuming a ketogenic diet.

- Carbohydrate forms a substantial chunk of most food that humans especially the Indian population consumes. Therefore, restricting carbohydrate by means of a ketogenic diet may not be practicable.

- Hence, a more feasible alternative would be intermittent fasting alone rather than routine carbohydrate restraint by way of a ketogenic diet. Both modalities seem to have comparable potential in interfering with the progression of the inflammatory process.

- Results obtained from the current study provide ample opportunity for future studies in human subjects. Since the current study is done in animals similar studies can be done in human subjects which would have widespread implications in management of NCDs which form a substantial burden in today's world both in developed and developing economies.

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9. ANNEXURES

ANNEXURE -1 ETHICAL CLEARANCE CERTIFICATE

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MDC / AH / _____ Date : 18-5-16

CERTIFICATE

This is to certify that the M.Sc / M.D / Ph.D / Research - Project entitled

"Effect of ketogenic diet, intermittent fasting and high carbohydrate diet on chronic inflammation in Male Wistar Rats

Submitted by Ms / Dr. Urmila A Kagal, Dept. of Pharmacology,

has been approved by the Institutional Animal Ethical Committee meeting

held on 14-5-2016 vide Resolution No. 7/A

Rats : 84 Male

Signatures :
& Name

Member Secretary
IAEC-JNMC

CPCSEA NOMINEE 14-5-2016
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ANNEXURE- 2

PUBLICATION

Biomedical & Pharmacology Journal, September 2019. Vol. **12**(3), p. 1457-1462

Effect of High Carbohydrate Diet on Complete Freund's Adjuvant Induced Inflammation in Rats

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Chronic low grade inflammation is an essential pathological feature of a variety of non-communicable diseases (NCDs). These diseases have now superseded infectious diseases where the burden of disease is concerned. One of the important modifiable factors contributing to chronic disease is food high in carbohydrate.This study was planned to study the role of high carbohydrate diet on a model of inflammation induced by Complete Freund's adjuvant(CFA) in male Wistar rats. Animals were divided into 3 groups of 10 rats each. Group I fed with standard dietserving as control; Group II fed with high carbohydrate diet(HCD) and Group III fed with standard diet serving as disease free normal group. CFAwas injected subcutaneously into the hind paw 4weeks after starting the diet into groups I and II only. Diet wascontinued for up to 21days after CFA injection. Digital plethysmometer measured the paw volume. Blood obtained before euthanasia served for estimating cytokines and oxidative stress parameters. A rise in paw edema was seen in control and high carbohydrate diet groups up to day 21. In the high carbohydrate groupthere were high serum cytokine levels and significant depletion of antioxidant enzymes. The authors conclude that, a high carbohydrate diet contributes significantly to the process of inflammation which has now been established as a significant factor in the causation of NCDs. Therefore it would be prudent to restrict carbohydrates in our diet.

Keywords: Carbohydrate, Complete Freund's Adjuvant, Cytokines, Inflammation, Oxidative stress.

Inflammation has been recognized for decades as a vital component of host defence. However, scientists have now come to realize that inflammation which serves a vital functionin tissue repair and immunosurveillance can be catastrophic in its chronic low grade form. A host of chronic non-communicable diseases (NCDs) such as type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), cardiovascular disease (CVD) and non-alcoholic fatty liver disease (NAFLD)have chronic low grade inflammation as an essential pathological feature[1].

Inflammation as described in terms of its classic features oftumor, rubor, dolor and calor is beneficial in the short term in bringing about tissue repair. Metabolic disorders where chronic inflammation plays a major role show that prolonged inflammation is not beneficial though the mediators involved in classic inflammation and in metabolic disease are the same[2].

Over the past 40 years the world has experienced a changing trend in disease patterns. The burden of disease which was dominated by infectious diseases in developing countries has now

been superseded by NCDs. According to the WHO, NCDs kill 17 million people before the age of 70 and 40 million people annually which acconut for 70% of deaths the world over. Over the next 20 years NCDs are going to cost the global economy US$47 trillionand are responsible for pushing millions of people to the brink of poverty[3,4].

Urbanization, increased marketing and affordability have led to the consumption of refined grains, sugar sweetened beverages, cakes, biscuits and confectionery which increase the risk of development of chronic disease. Modern society has become unhealthy when compared to our healthy and robust hunter gatherer ancestors as a result of lack of physical exercise and consumption of junk food which is highly processed[4,5,6].

The susceptibility to these diseases is determined by an amalgam of genetic and environmental factors. Industrializing countries with a growing economy have a large population of people who have migrated from rural to urban areas as a result of which, their lifestyles and diets have changed. These changes may have unveiled a susceptibility to these new diseases. Modern society has started to follow diets rich in sugar and refined food stuffs and poor in dietary fiber content[7].

From the literature reviewed it can be concluded that, one of the important modifiable risk factors contributing to chronic disease is food which is high in carbohydrate and that chronic inflammation happens to be an essential pathophysiological feature of chronic NCDs. Thereforethe objective of this study was toevaluate the role of high carbohydrate diet in a model of inflammation induced by Complete Freund's adjuvant(CFA) in male Wistar rats.

MATERIALS AND METHODS

Complete Freund's adjuvant of 1mg/ ml concentration was purchased from Sigma Aldrich, Saint Louis, Missouri, USA. Digital plethysmometer was purchased from Orchid Scientific and Innovative India Pvt. Ltd. Nashik, Maharashtra, India. Colorimetric kit for measuring Thiobarbituric acid reactive substances (TBARS) was purchased from Bioassay Systems, USA. ELISA kits for measuring catalase and superoxide dismutase (SOD) were purchased from MyBioSource, Inc, San Diego, CA, USA. ELISA kits for measuring tumor necrosis factor alpha (TNF á) and interleukin 1 beta (IL-1â) were purchased from Krishgen Biosystems, Mumbai, India.

Diet was produced from locally available ingredients except zero carbohydrate whey protein manufactured by Isopure which was purchased from Amazon.

Animals

Adult male Wistar rats (weighing 150-200g) obtained from Central Animal Facility of the institution were used in the present study. Animals were housed under standard conditions. The animal experiment was reviewed and approved by the Institutional Animal Ethics Committee (Letter no.7/A dated 18/05/2016). Animal handling and experiments were performed according to the guidelines put forward by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Animal Procedures

The animals were divided into 3 equal groups (n = 10 per group).

Group I- Rats fed with standard diet injected with CFA serving as control

Group II- Rats fed with high carbohydrate diet (HCD)

Group III - Rats fed with standard diet not injected with CFA serving as disease free normal group

The respective diets (composition elaborated in Table 1) were started 4weeks before induction of inflammation and continued throughout the study period. All diets were fed on *ad libitum* basis.Diets were prepared as per the principles mentioned in the literature[8,9].

Induction of inflammation

Inflammation was induced by subcutaneously injecting 0.1ml of CFA (1mg/ml concentration) at the back surface of right hind paw. CFA was injected into animals belonging to Group I and II, but not Group III. The diet was continued for 21 days following the injection of CFA. Paw volume of injected hindpaw was measured on days 0, 3, 7, 14 and 21using a digital plethysmometer[10]

Serum analysis

Blood was obtained by cardiac puncture after anesthetizing the animals using intraperitoneal thiopentone on day 21 following which animals were euthanized by thiopentone overdose. Blood

was allowed to clot, following which serum was separated by centrifuging it at 2000 rpm for 15 min and the obtained serum was used for estimation of cytokines and oxidative stressparameters.

Assessment of Inflammation and Oxidative stress

Estimation of inflammatory mediators

The serum levels of inflammatory mediators like TNF áand IL-1â were estimated quantitatively using ELISA kits as per the directions mentioned in the manufacturer's protocol.

Estimation of Oxidative stress parameters

The serum levels of TBARS were measured using a colorimetric kit and antioxidant enzymes namely SOD and catalase were estimated quantitatively using ELISA kits as per the directions mentioned in the manufacturer's protocol.

Data analysis

The data was analyzed using statistical software Graph Pad Prism (GraphPad Software, Inc. La Jolla, California, USA). To assess the differences between the groups One-way analysis of variance (ANOVA) was carried out which was followed by Dunnett's post hoc analysis. A p value of < 0.05 was considered significant.

Table 1. Composition of diets

Constituents / 100g of diet	Standard diet	High carbohydrate diet
Wheat flour	50g	-
Maida	-	32g
Sugar (Sucrose)	-	32g
Whey protein	25g	26g
Ghee	05g	05g
Bran	20g	05g

RESULTS

The present study was planned to evaluate the role of high carbohydrate diet in a model of inflammation induced by CFA in male Wistar rats. All results have been expressed as mean ± standard error of mean (SEM).

Effect of high carbohydrate diet on CFA induced paw edema

A rise in paw edema was seen both in control and high carbohydrate diet groups as compared to day 0. There was a slight fall on day 7 in both groups which reflects the natural course of paw edema development following CFA injection following which there was a rise up to day 21. But, the rise in paw edema from day 3 onwards was significantly more in the HCD group compared to the control group as depicted in Table 2.

Effect of high carbohydrate diet on inflammatory cytokines

Analysis of inflammatory cytokines namely TNF áand IL-1âin the serum revealed that, in the HCD group there was a significant increase in levels of both cytokines compared to the control group as depicted in Table 3.

Effect of high carbohydrate diet on oxidative stress parameters

Analysis of TBARS and antioxidant enzymes in the serum revealed that, in the HCD group there was a significant increase in the level of TBARS and significant depletion of antioxidant enzymes superoxide dismutase and catalase compared to control group as depicted in Table 4.

Table 2. Effect of high carbohydrate diet on Complete Freund's adjuvant (CFA) induced paw edema

Mean ± SEM(ml)	Control	HCD	Normal	F value	P value
Day 0	1.071 ± 0.037	1.08 ± 0.042	1.057 ± 0.034	0.091	0.9128
Day 3	1.729 ± 0.041	2.162 ± 0.043 ****	1.124 ± 0.026****	186.9	< 0.0001
Day 7	1.518 ± 0.042	1.696±0.067*	1.16 ± 0.024****	31.9	< 0.0001
Day 14	1.793 ± 0.038	2.2±0.059****	1.032 ± 0.027****	183.4	< 0.0001
Day 21	2.095 ± 0.038	2.270 ± 0.053**	1.185 ± 0.02****	213.2	< 0.0001

Analysis by ANOVA followed by Post Hoc Dunnett's test.
* indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001
SEM: Standard error of mean; HCD: High carbohydrate diet

DISCUSSION

The present study was planned to evaluate the role of high carbohydrate diet on a model of inflammation induced by CFA in male Wistar rats. The results of the present study provethat, feeding a high carbohydrate diet over a period of 7 weeks adds to the inflammation produced by CFA, which is sustained over a period of 21days. Apart from this, there is arise in inflammatory cytokines, susceptibility to lipid peroxidation and depletion of antioxidant defenses in rats fed a high carbohydrate diet.

The strengths of the present study are the with respect to the ingredients of the high carbohydrate diet and the parameters evaluated in the study.

The key ingredients used in the high carbohydrate diet are sucrose (which contains glucose and fructose) and maida (highly refined wheat flour) which are being extensively in the present day and age especially in the production of junk food which is a major contributor to NCDs like T2DM, obesity and ischaemic heart disease. Hence, conclusions drawn from this study are relevant to the human population though, the study has been done in rats.

Another strength of the study is the fact that, it has not just confined itself to the measurement of a parameter like paw edema which is visible to the naked eye but, has taken the study to a higher level by evaluating the effects of a high carbohydrate diet on markers like inflammatory cytokines and oxidative stress parameters.

In the present study, levels of serum cytokines, namely TNF-á and IL-1â have been found to be increased in the HCD group. Similar findings were also reported by a study which evaluated the effect of carbohydrates like fructose and sucrose on the same cytokines[11].

The elevation of cytokines is caused not just by sugar, but also a refined carbohydrate like bread which contains maida which was proven by a study where in white breadwas found to acutely activate nuclear factor kappa light chain enhancer of activated B cells (NF- êB)[12]. NF-kB is a transcription factor which controls the expression of genes involved in inflammation. It is important to note that, NF-kB increases the synthesis of pro-inflammatory cytokines namely IL-1, IL-6 and TNF- alpha[13].

Apart from increased susceptibility to lipid peroxidation as shown by the increased

Table 3. Effect of high carbohydrate diet on inflammatory cytokines

Mean ± SEM	Control	HCD	Normal	F value	P value
Serum IL-1â (pg/ml)	652.5 ± 9.73	945.1± 10.89****	113.7 ±2.62****	2421	< 0.0001
Serum TNF á (pg/ml)	37.54 ± 2.96	48.75 ± 2.47**	15.71 ± 0.65 ****	55.22	< 0.0001

Analysis by ANOVA followed by Post Hoc Dunnett's test
* indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001
SEM: Standard error of mean; HCD: High carbohydrate diet; IL-1â: Interleukin 1 beta;
TNF á:Tumor necrosis factor alpha

Table 4. Effect of high carbohydrate diet on oxidative stress parameters

Mean ± SEM	Control	HCD	Normal	F value	P value
Serum SOD (U/ml)	140 ±1.33	91.49 ± 1.37 ***	231.7 ± 14.06****	75.51	< 0.0001
Serum catalase (pg/ml)	60.87 ± 2.24	25.35 ± 3.835 ****	244.3 ± 2.12 ****	1707	< 0.0001
Serum TBARS(iM)	14.79 ± 0.90	18.11 ± 0.25 **	4.480 ± 0.46 ****	136.7	< 0.0001

Analysis by ANOVA followed by Post Hoc Dunnett's test
* indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001
SEM: Standard error of mean; HCD: High carbohydrate diet; SOD: Superoxide dismutase; TBARS:Thiobarbituric acid reactive substances

serum TBARS levels, the present study also found significant depletion of antioxidant enzymes namely SOD and catalase in theHCD group. Similar findings were found in a study, in which rats were fed high sucrose diet for 2 weeks.Significant increase in the plasma TBARS levels and decreased SOD levels were found in the hearts of the rats which were fed a high sucrose diet[14].

Apart from the mechanisms documented in the present study, there are other mechanisms by which carbohydrates can contribute to inflammation and oxidative stress as documented in the literature.

Fructose can induce an accumulation of advanced glycation end-products and the oxidative degradation of fructose adducts can lead to production of free radicals[15].

A diet rich in sucrose / fructose can induce inflammation by altering the gut microbiota. There is ultimately an increase in gram negative bacteria leading to excess lipopolysaccharide (LPS) release. This LPS release causes an increase in mRNA expression levels of toll like receptors (TLRs) TLR2 and TLR4[11]. TLR4 binds to LPS of bacterial cell walls of gram negative bacteria. This binding activates a signaling pathway, leading to activation of the NF-êB pathway which in turn activates cytokines, chemokines and other effectors of innate immunity[16].

An acutely high glucose causes alterations in osmolarity leading to activation of NF-kB. Exposure to high glucose for more prolonged times causes changes in antioxidant defences and activation of protein kinase C (PKC), which potentiates activation of NF-kB[17].

CONCLUSION

From the results of the present studyit can be concluded that, a high carbohydrate diet contributes significantly to the process of inflammation by causing a rise in inflammatory cytokines, increased susceptibility to lipid peroxidation and depletion of antioxidant defenses. Inflammation has now been firmly established as an essential pathophysiological component in the pathogenesis of non - communicable diseases. Hence, it would be prudent to restrict carbohydrates in our diet.

ACKNOWLEDGEMENT

NIL

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ORIGINAL ARTICLE

Effect of Ketogenic Diet and Intermittent Fasting on Complete Freund's Adjuvant Induced Inflammation in Rats

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Abstract:

Background: Chronic low grade inflammation is an essential pathological feature of various Noncommunicable Diseases (NCDs). Carbohydrate rich food an important modifiable risk factor contributing to NCDs can be restricted by Ketogenic Diet (KD) or Intermittent Fasting (IF). So the present study was taken up to evaluate the role of the above interventions in inflammation induced by Complete Freund's Adjuvant (CFA) in male Wistar rats. *Aim and Objectives:* To evaluate the role of KD and IF in a model of inflammation induced by CFA in rats. *Material and Methods:* Animals were divided into 4 equal groups of 10 rats each. Groups were based on diet given. Group I - Control fed with standard diet; Group II - KD; Group III - IF and Group IV- Disease free group on standard diet. CFA injection into the hind paw 4 weeks after starting the diet was made in all groups except in group IV after which serum cytokines and oxidative stress markers were measured on day 21. *Results:* KD and IF groups showed significantly lower inflammation in terms of paw edema volume and serum cytokines namely TNF-α and IL-1β as compared to the control group. The KD and IF groups also showed lower oxidative stress in terms of lower serum Thiobarbituric Acid Reactive Species (TBARS) and less depletion of antioxidant enzymes namely superoxide dismutase and catalase as compared to control group. *Conclusion:* The present study proves that it is possible to lower inflammation and hence prevent NCDs by IF and KD. Since the impact of both interventions on inflammation is similar, IF may be preferable to KD because, carbohydrate is present in a sizeable amount in most foodstuffs that we consume and daily restriction of carbohydrate in the form of KD may not be a feasible option. Hence, IF alone may be a better option than daily carbohydrate restriction in the form of KD.

Keywords: Ketogenic diet, Intermittent Fasting, Inflammation, Freund's Adjuvant

Introduction:

Inflammation can be looked upon as a friend and a foe which on the one hand serves as a vital component of host defense while on the other hand, in its chronic low grade form is an essential pathological feature of a host of Noncommunicable Diseases (NCDs) of a chronic nature such as Type 2 Diabetes Mellitus (T2DM), Metabolic Syndrome (MetS), Cardiovascular Disease (CVD) and Non-Alcoholic Fatty Liver Disease (NAFLD) [1]. Inflammation as described in terms of its classic features namely tumor, rubor, dolor and calor may be beneficial in the short term in bringing about tissue repair. Metabolic disorders where in chronic inflammation plays a major role, bear witness to the fact that, prolonged inflammation is not beneficial although, the mediators involved in classic inflammation and those observed in metabolic disease may be the same. Hence, a new term called meta-flammation (metabolically triggered inflammation) has been coined [2].

Over the past 40 years the world has experienced a changing trend in disease patterns and threats to global health. The burden of disease which was predominated by infectious diseases in the middle and low income group countries has now been superseded by NCDs which pose a threat not just to the developed countries but to the world as a whole. NCDs which include cancer, diabetes, chronic obstructive pulmonary disease, cardiovascular disease and mental health conditions have replaced undernourishment, Tuberculosis (TB), malaria and HIV which were threats to global health in the past. According to the WHO, NCDs kill 17 million people before the age of 70 and 40 million people annually which amounts to 70% of deaths the world over. Over the next 20 years NCDs are going to cost the global economy US$47 trillion and are responsible for pushing millions of people to the brink of poverty [3, 4].

Factors such as urbanization, increased marketing and affordability have led to the consumption of refined grains, sugar sweetened beverages, cakes, biscuits and confectionery which increase the risk of development of chronic disease. In today's modern society human beings have become unhealthy as a result of lack of physical activity and consumption of processed junk food. This is in stark contrast to our ancestors who were healthy and robust because of the fact that, they were hunter gatherers and had to walk for miles together every day in search of food [4-6].

The susceptibility to NCDs is determined by an amalgamation of genetic and environmental factors. Industrializing countries with a growing economy have a large population of people who have migrated from rural to urban areas as a result of which, their lifestyles and diets have changed in comparison to their diets and lifestyles prior to migration. These changes may have unveiled a susceptibility to these diseases. Modern society which has emerged from an improved economy has started to consume diets rich in sugar and refined food stuffs and poor in dietary fiber content [7].Government of India has launched a programme called National Programme for Prevention and Control of Cancer, Diabetes, Cardiovascular disease and Stroke (NPCDCS) after taking into consideration the growing burden of diabetes and other NCDs. As per this programme, lifestyle management of which diet is an integral part forms the cornerstone of both prevention and management of NCDs [8].

From the literature reviewed it can be concluded that, one of the important modifiable risk factors contributing to chronic disease is food which is high in carbohydrate and that chronic inflammation happens to be an essential pathophysiological feature of chronic NCDs. Therefore, a cost-effective and logical approach to reduce the risk of chronic disease would be to cut down carbohydrate by either Ketogenic Diet (KD) or Intermittent Fasting (IF). Hence, this study was taken up with the objective of evaluating the role of KD and IF in a model of inflammation induced by Complete Freund's Adjuvant (CFA) in male Wistar rats.

Material and Methods:

CFA of 1mg/ml concentration was purchased from Sigma Aldrich, Saint Louis, Missouri, USA. Digital plethysmometer was purchased from Orchid Scientific and Innovative India Pvt. Ltd. Nashik, Maharashtra, India. Colorimetric kit for measuring Thiobarbituric Acid Reactive

Substances (TBARS) was purchased from Bioassay Systems, USA. ELISA kits for measuring catalase and Superoxide Dismutase (SOD) were purchased from MyBioSource, Inc, San Diego, CA, USA. ELISA kits for measuring tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1β) were purchased from Krishgen Biosystems, Mumbai, India. Diet was produced from locally available ingredients except zero carbohydrate whey protein manufactured by Isopure which was purchased from Amazon.

Animals:

Adult male Wistar rats (weighing 150-200g) obtained from Central Animal Facility of the institution were used in the present study. Animals were housed under standard conditions. The animal experiment was reviewed and approved by the Institutional Animal Ethics Committee (Ref. no.7/A dated 18/05/2016). Animal handling and experiments were performed according to the guidelines put forward by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Animals Grouping:

The animals were divided into 4 equal groups (n = 10 animals per group) [9]

Group I- Rats fed with standard diet serving as control

Group II - Rats fed with KD and injected with CFA

Group III - Rats which have been fasted on alternate days and fed the standard diet on non-fasting days serving as IF group

Group IV - Rats fed with standard diet serving as disease free normal group

The respective diets (composition elaborated in Table 1) were started 4 weeks before induction of inflammation and continued throughout the study period. All diets were fed on *ad libitum* basis [10].

Table 1: Composition of Diets

Constituents / 100g of diet	SD	KD
Wheat flour	50g	01g
Whey protein	25g	20g
Coconut oil	-	09g
Ghee	05g	60g
Bran	20g	10g

SD-Standard Diet, KD-Ketogenic Diet

Induction of inflammation:

Inflammation was induced by subcutaneously injecting 0.1ml of CFA (1mg/ml concentration) at the back surface of right hind paw. CFA was injected into animals belonging to Groups I, II and III, but not Group IV. The diet was continued for 21 days following the injection of CFA. Paw volume of injected hind paw was measured on day 0, 3, 7, 14 and 21 using a digital plethysmometer [11].

Serum Analysis:

Blood was obtained by cardiac puncture after anesthetizing the animals using intraperitoneal thiopentone on day 21 following which animals were euthanized by thiopentone overdose. Blood was allowed to clot, following which serum was separated by centrifuging it at 2000 rpm for 15 min and the obtained serum was used for estimation of cytokines and oxidative stress parameters.

Assessment of Inflammation and Oxidative Stress:

Estimation of Inflammatory Mediators:

The serum levels of inflammatory mediators like Tumor Necrosis Factor Alpha (TNFα) and Interleukin 1 beta (IL-1β) were estimated quantitatively using ELISA kits as per the directions mentioned in the manufacturer's protocol.

Estimation of Oxidative Stress Parameters:

Serum levels of TBARS were measured using a colourimetric kit and antioxidant enzymes namely SOD and catalase were estimated quantitatively using ELISA kits as per the directions mentioned in the manufacturer's protocol.

Data Analysis:

The data was analyzed using statistical software Graph Pad Prism (GraphPad Software, Inc. La Jolla, California, USA). To assess the differences between the groups One-way Analysis of Variance (ANOVA) was carried out which was followed by Dunnett's *post hoc* analysis. *P* value of < 0.05 was considered significant.

Results:

The present study was planned to evaluate the role of KD and IF in a model of inflammation induced by CFA in male Wistar rats. All results have been expressed as Mean ± Standard Error of Mean (SEM).

Effect of various diets on CFA induced paw edema:

A rise in paw edema was seen in all groups except disease free normal group as compared to day 0. There was a slight fall in paw edema on day 7 in all groups injected with CFA which reflects the natural course of paw edema development after CFA injection following which there was a rise upto day 21. But, the rise in paw edema from day 3 onwards was significantly lower in the KD and intermittent fasting groups (Table 2, Fig.1).

Table 2: Effects of KD and IF on CFA Induced Paw Edema

Mean ± SEM (ml)	Control	KD	IF	Normal	F value	P value
Day 0	1.071 ± 0.037	1.025 ± 0.03	0.983 ± 0.028	1.057 ± 0.034	1.419	0.2532
Day 3	1.729 ± 0.041	1.432 ± 0.037****	1.516 ± 0.022***	1.124 ± 0.026****	57.45	**< 0.0001**
Day 7	1.518 ± 0.042	1.263 ± 0.033****	1.342 ± 0.034**	1.16 ± 0.024****	19.24	**< 0.0001**
Day 14	1.793 ± 0.038	1.378 ± 0.027****	1.443 ± 0.025****	1.032 ± 0.027****	108	**< 0.0001**
Day 21	2.095 ± 0.038	1.162 ± 0.033****	1.323 ± 0.041****	1.185 ± 0.02****	163.5	**< 0.0001**

KD-Ketogenic Diet, IF-Intermittent Fasting, indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

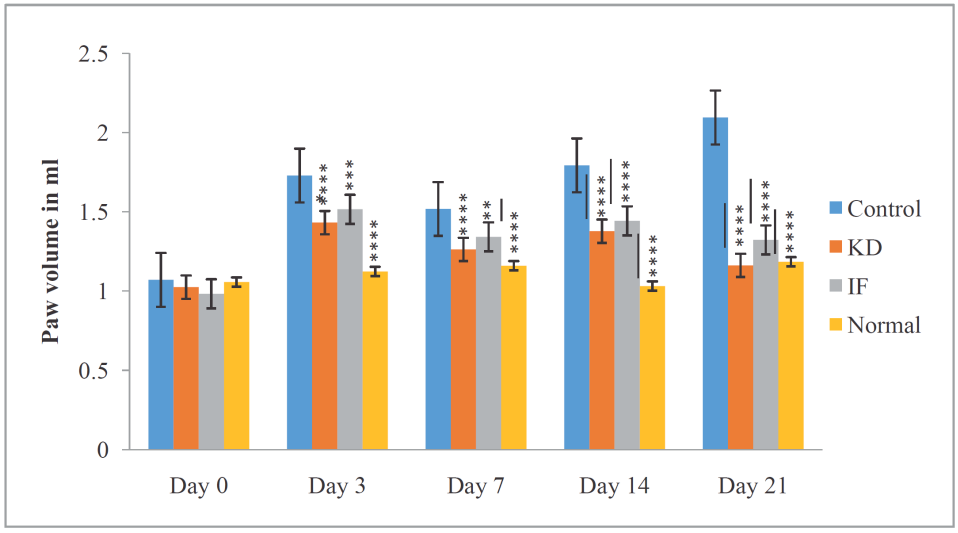

Fig. 1: Effect of various diets on CFA induced paw edema

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

Effect of various diets on serum cytokine levels:

Analysis of cytokines namely TNF-α and IL-1β in the serum revealed that, in the KD and IF groups there was a significant depression in the levels of these cytokines as compared to control group (Table 3, Figs. 2 and 3).

Effect of various diets on oxidative stress parameters:

Analysis of TBARS and antioxidant enzymes in the serum revealed that, in the KD and IF groups there was a significant decrease in the level of TBARS (Table 4, Fig. 4), and significantly less depletion of antioxidant enzymes namely super-oxide dismutase and catalase compared to control group (Table 4, Figs. 5 and 6).

Table 3: Effects of KD and IF on Inflammatory Cytokines

Mean ± SEM	Control	KD	IF	Normal	F value	P value
Serum Il–1β (pg/ml)	652.5 ± 9.73	167.9 ± 2.5****	256.4 ± 8.28****	113.7 ± 2.62****	1345	**< 0.0001**
Serum TNF α (pg/ml)	37.54 ± 2.96	23.08 ± 1.33****	21.51 ± 1.36****	15.71 ± 0.65****	26.74	**< 0.0001**

KD-Ketogenic Diet, IF-Intermittent Fasting, indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

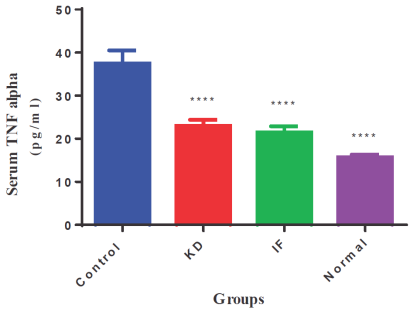

Fig. 2: Effect of various diets on serum TNF alpha

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

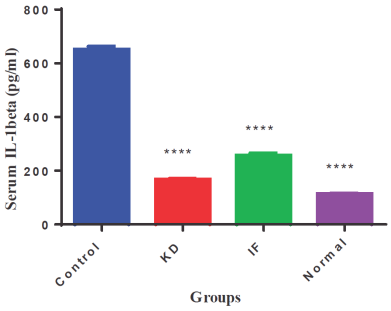

Fig. 3: Effect of various diets on serum IL-1 beta

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

Table 4: Effects of KD and Intermittent Fasting on Oxidative Stress Parameters

Parameters	Control	KD	IF	Normal	F value	P Value
SOD (U/ml)	140 ± 1.33	182.4 ± 4.79**	183.1± 3.77***	231.7 ± 14.06****	23.74	**< 0.0001**
Catalase (pg/ml)	60.87 ± 2.24	118.9 ± 3.26****	164.3 ± 2.96****	244.3 ± 2.12****	827	**< 0.0001**
TBARS (µM)	14.79 ±0.9	8.2 ± 1.06****	10.99 ±0.87**	4.48 ±0.46****	25.86	**< 0.0001**

KD-Ketogenic Diet, IF-Intermittent Fasting, indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

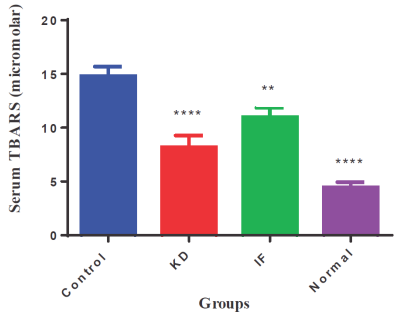

Fig. 4: Effect of various diets on serum TBARS

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

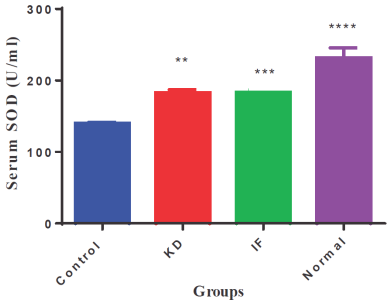

Fig. 5: Effect of various diets on serum SOD

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

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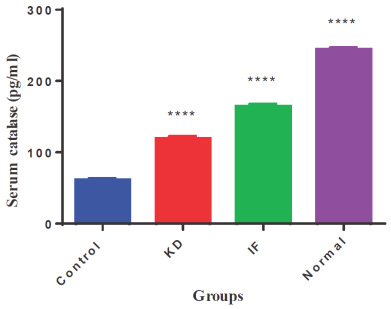

Fig. 6: Effect of various diets on serum catalase

*KD-Ketogenic Diet, IF-Intermittent Fasting, * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; **** indicates P < 0.0001*

Discussion:

The present study was planned to evaluate the role of KD and IF in a model of inflammation induced by CFA in male Wistar rats.

The results so far obtained, indicated that KD and IF can lower inflammation by reducing paw edema, lowering levels of inflammatory cytokines, reducing susceptibility to lipid peroxidation and preventing significant depletion of antioxidant defenses.

As mentioned earlier, one of the modifiable risk factors contributing to chronic disease is food which is rich in carbohydrate, and hence a rational approach to reduce the risk of chronic NCDs would be to restrict carbohydrate by either KD or IF. In the present study, the role of these two interventions has been evaluated on CFA induced inflammation, since the inflammation induced by this agent is long lasting and thus, the effect of the interventions on edema can be measured consistently over a period of time.

Studies done in the past have evaluated the role of KD and IF over a very short period of 24-48 hours [10, 12-14] in contrast to the present study where in the effect of these interventions has been evaluated over a period of 21 days. The study has delved into the effects of these interventions not just on paw edema which is a gross parameter but also its effects on molecular markers like inflammatory cytokines and oxidative stress parameters.

KDs are characterized by high-fat, moderate protein and low-carbohydrate components, resulting in increased fat metabolism and limited metabolism of carbohydrates and proteins. As a result of this, a metabolic state develops where in there are increased fat-derived ketone bodies and decreased levels of glucose in the blood. This metabolic state first described by Hans Krebs as physiological ketosis is a metabolic state in which the body obtains its energy from the metabolism of ketone bodies, as opposed to what occurs in glycolysis, where glucose is the main energy source. Ketosis may be achieved through periods of fasting or by reducing the intake of carbohydrates in the diet [15].

This physiological ketosis differs from pathological ketoacidosis a complication of diabetes mellitus, where in blood ketone levels can exceed 20mmol/l with a simultaneous lowering of blood pH. In contrast to this, in physiological ketosis blood ketone levels reach a maximum level of 7/8 mmol/l and there is no change in pH [16].

Various fasting regimens have been described and experimented with in animal and human studies. The ones commonly used are total fasting on alternate days where in no energy containing foods or beverages are allowed on fasting days with *ad libitum* feeding on eating days; 5:2 diet involves severe energy restriction for two nonconsecutive days per week and *ad libitum* eating for the

remaining 5 days. Time-restricted feeding allows for energy intake at liberty but, within precise frames of time during the day resulting in regular, extended fasting intervals [17].

Inflammation at the gross level in the present study has been measured using a digital plethysmometer after inducing inflammation using CFA which shows a significant reduction in inflammation in the interventional groups over several time points the last measurement being recorded on day 21. To date, only a single study has shown reduction in CFA induced inflammation in KD fed rats. But, the study has measured inflammation only after a period of 48 hours in contrast to the present study, which has measured inflammation over several time points and has shown the beneficial effects of KD and IF over a period of 21days [10].

In the present study, levels of serum cytokines, namely TNF-α and IL-1β have been shown to be reduced in the interventional groups. The findings of this study are in agreement with findings of other studies which have also shown reduction in serum cytokine levels but, the models of inflammation used are different from those used in the present study [12-13].

In a study involving Spinal Cord Injury (SCI) in rats, it was found that KD attenuates the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway 4 weeks post-injury, possibly resulting in the observed reduced expression of proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ). Apart from this, it was found that, SCI significantly increased nuclear factor erythroid 2-related factor 2(Nrf2) levels with a greater increase in the KD group compared to standard diet group [18].

The present study also has measured various oxidative stress parameters like TBARS and antioxidant enzymes namely SOD and catalase in the serum. The results of the present study have shown reduced susceptibility to lipid peroxidation as measured by serum TBARS and preservation of antioxidant defenses in the interventional groups as compared to the control group. TBARS is considered an important marker of oxidative stress [19].

Caloric restriction decreases mitochondrial generation of Reactive Oxygen Species (ROS) by enhancing the activity of Uncoupling Proteins (UCP). UCP span the inner membrane of the mitochondria allowing the leakage of protons from the intermembrane space to the matrix. This mechanism causes the electrochemical gradient (proton motive force) to dissociate or uncouple from ATP generation. This uncoupling serves to reduce the mitochondrial membrane potential and decreases the production of ROS [20, 21].

Caloric restriction has been found to reduce NFκB levels (probably a Sirt1-dependent process), block synthesis of interleukins and TNFα and suppress the activity of COX-2 and iNOS. Transcription factors NF-kB and Nrf2 are stimulated by lipid peroxides, ROS and reactive nitrogen species [20, 21].

The transcription factor NF-kB promotes immunity by controlling the expression of genes involved in inflammation. Among the important pro-inflammatory cytokines, the synthesis of which is controlled by NF-kB are IL-1, IL-6 and TNF- alpha. NF-kB signaling in several types of cells contributes to the pathology of metabolic disorders notable ones being obesity, type 2 diabetes mellitus and atherosclerosis [22].

Nrf2 is a transcription factor modulating adaptive responses to intrinsic and extrinsic cellular stresses. Among the multiple cellular functions of Nrf2 the ones of interest are - provides direct antioxidants, codes for enzymes that directly nullify oxidants, enhances the recognition, repair and removal of damaged proteins and suppresses inflammation mediated by cytokines [17, 18].

Similar to caloric restriction KD also stimulates the cellular endogenous antioxidant system. Particularly important is the activation of Nuclear Factor Erythroid-derived 2 (NF-E2)-related factor 2 (Nrf2) since it is the major inducer of detoxification genes. The ketone body, beta hydroxybutyrate, is an endogenous inhibitor of Histone Deacetylases (HDACs) belonging to class I and class IIa. Inhibition of HDACs results in upregulation of transcription of various detoxifying genes, of relevance being catalase, mitochondrial SOD and metallothionein 2 which help in counteracting oxidative stress. Other mechanisms explaining the antioxidant property of KD include modulation of the NAD+/NADH ratio which offers protection against ROS and the increased competence of electron transport chain through the manifestation of uncoupling proteins [23].

In spite of robust advances in the process of drug discovery using sophisticated state of the art techniques, the morbidity caused by adverse drug reactions continues to cause human suffering. Hence, there has been a worldwide emergence in medical centres focusing on complementary and integrative medicine leveraging on the wisdom of the ages that, nutrition, exercise, yoga and acupuncture have an important role to play in averting human suffering. But this argument becomes meaningless scientifically, unless substantiated by evidence from animal data and human studies which offer insights into the cellular and molecular mechanisms of action of these traditional forms of healing [24].

Conclusion:

The findings of the present study prove that it is possible to modify the process of inflammation by IF and KD. Since, carbohydrate is present in a sizeable amount in most foodstuffs that we consume; daily restriction of carbohydrate in the form of KD may not be a feasible option. Hence, IF alone may be a better option than daily carbohydrate restriction in the form of KD since both interventions have been found to have a similar impact in reducing the process of inflammation.

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