**JKIMSU, Vol. 8, No. 4, October-December 2019** **ISSN 2231-4261**

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*

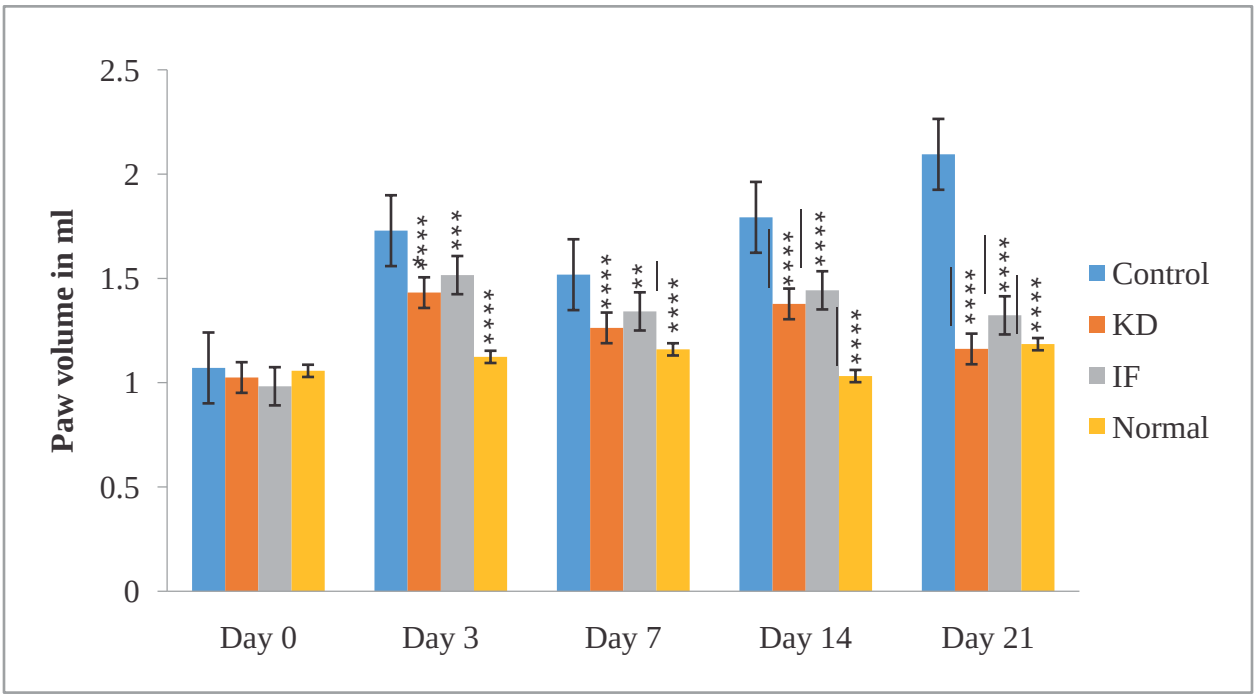


**Fig. 1: Effect of various diets on CFA induced paw edema**

*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*

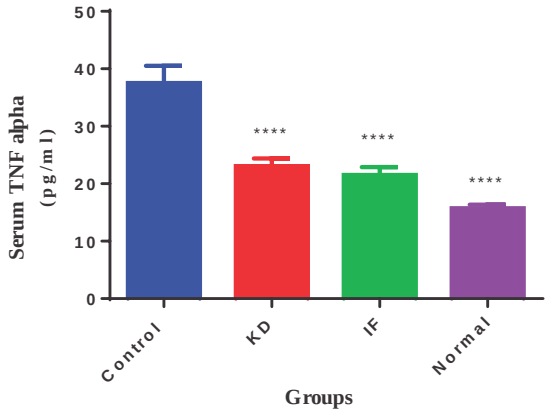


**Fig. 2: Effect of various diets on serum TNF alpha**

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

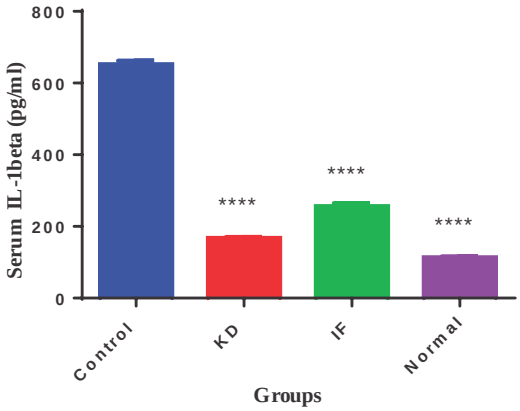


**Fig. 3: Effect of various diets on serum IL-1 beta**

*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*

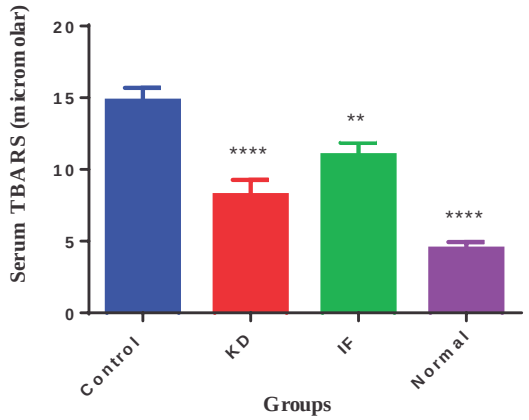


**Fig. 4: Effect of various diets on serum TBARS**

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

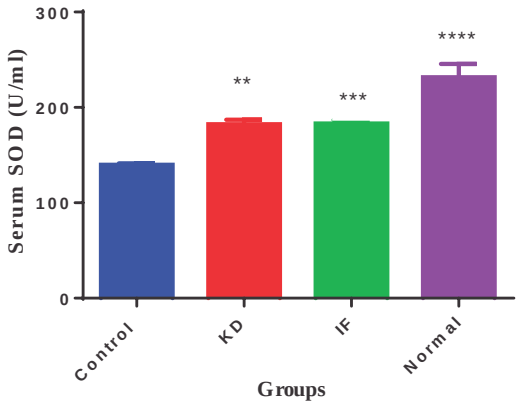


**Fig. 5: Effect of various diets on serum SOD**

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

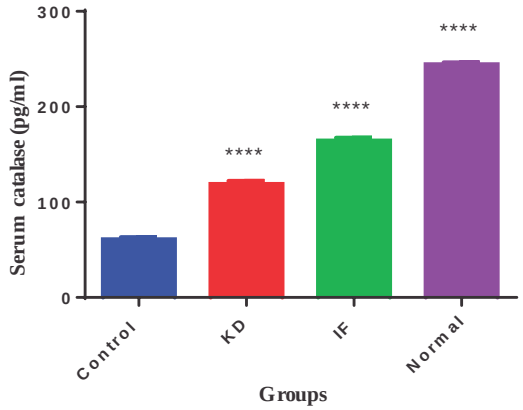


**Fig. 6: Effect of various diets on serum catalase**

*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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