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Impact of Calorie Restriction on Expression of Apoptotic and Inflammatory Genes in Diabetic Rat Model

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Abstract: Calorie restriction, the nutritional intervention which reduces calorie intake without incurring malnutrition has been reported to reduce inflammation and the expression of inflammatory genes. Diabetes is associated with low grade inflammation state. The effect of calorie restriction on the expression of apoptosis and inflammatory genes in male Wistar rats induced with diabetes was investigated and compared with the control group. The lung cells were harvested, isolated, incubated and observed for the formation of epithelial and/or fibroblast monolayer. The extracted total RNA was reverse transcribed into cDNA, and real-time PCR was carried out. The alveolar cells were cultured in growth media simulating calorie restriction and hyperglycemia and were observed for phenotypic changes and gene expression. Changes in morphology were observed in cells cultured in high glucose, resembling fibroblast phenotype, 10 days post culture. The expression levels of apoptotic genes such as bax, TNF-α, caspase 3 and caspase 8 and pro-inflammatory genes such as Rantes, iNOS, MCP-1, MIP-2 and IL-1 were substantially up regulated in the diabetes induced alveolar epithelial cells compared to that of non-diabetes induced alveolar epithelial cells suggesting that diabetes induced pathological changes in the lung were associated with the induction of apoptosis and inflammation. Overall, this study provides evidence that a planned calorie restriction in diabetes significantly down-regulates the expression levels of inflammatory and apoptotic genes, which is likely to improving metabolic outcomes in type 2 diabetes.

Keywords: *Calorie Restriction, Diabetes, Apoptotic and Inflammatory Genes.*

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I. INTRODUCTION

Calorie restriction (CR) is a dietary regimen involving reduced calorie intake without incurring malnutrition or a reduction in the essential nutrients [1]. Adequate nutriture is designed into CR regimens to avoid the confounding effects of malnutrition. This regimen is well known for its importance in the well-being of organisms extending from worms to mammals and has been widely studied and found to be an effective experimental strategy for increasing the longevity in diverse animals ranging from unicellular yeast to multicellular rhesus monkeys, including humans [2]. Numerous studies have revealed that CR induces several benefits that are directly linked to extending the lifespan [3]. These benefits include CR acting as an effective intervention strategy for combating and preventing carcinogenesis in experimental models [4]. Also, it has been found to reduce the oxidative stress-induced associated metabolic disorders including diabetes, retard age-associated changes such as reducing the onset of cardiovascular disorders in non-obese individuals. At the tissue level, CR is found to lower serum glucose and cholesterol levels, increase insulin sensitivity, as well as decrease oxygen consumption, thus contributing to cell longevity. It is established that CR blunts the oxidative stress-induced cell damage thus promoting cell survival. The highest attenuation of CR against oxidative damage is in the brain, heart and skeletal muscle which are composed of long-lived postmitotic cells [3].

Due to the changes in lifestyle, metabolic disorders are on the rise globally and visceral fat has been identified as the primary indicator of these metabolic syndromes. CR has been shown to decrease adiposity, particularly visceral fat in humans and decreasing the visceral fat by way of CR is associated with an increased mean and maximum lifespan [5, 6]. This is because weight loss by CR in obese individuals has

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been shown to result in decreased risk of CVD and the weight loss should be targeted at reducing abdominal fat [7]. Moderate CR reduces or prevents the incidence of obesity, type 2 diabetes, and CVD [8]. A short-term CR study conducted in overweight men and women resulted in reduced visceral fat, decreased insulin resistance, lower metabolic rate, body temperature, and oxidative stress [8].

Table 1 List of Primers Used

Genes	Forward Primers	Reverse Primers
18S rRNA	TCCCAGTAAGTGCGGGTCATA	CGAGGGCCTCACTAAACCATC
Bax	TGGCAGCTGACATGTTTGCT	TTTAGTGCACAGGGCCTTGAG
Rantes	ACTCCCTGCTGCTTTGCCTACC	TTGGCGGTTCCTTCGAGT
TNF-α	CCCATGTTGTAGCAAACCCTC	TATCTCTCAGCTCCACGCCA
INOS	GTCACCTATCGCACCCGAGATG	GCCACTGACACTCCGCACAAAG
Caspase 3	TGTGAGGCGGTTGTAGAAGAGTTTC	GCACACCCACCGAAAACCAG
Caspase 8	GTGTGGGGTAATGACAATCTCG	CTCTTCAAAGGTCGTGGTCAAA
MCP-1	TCACGCTTCTGGGCCTGTTG	CAGCCGACTCATTGGGATCATC
MIP-2	GGCAAGGCTAACTGACCTGGAAAG	CACATCAGGTACGATCCAGGCTTC
IL-1	AGCTCCACGGGCAAGACATAGG	GGATGGCTTCCAAGCCCTTGAC
IL-10	GGCTCAGCACTGCTATGTTGCC	AGCATGTGGGTCTGGCTGACTG

According to the latest International Diabetes Federation (IDF) Diabetes Atlas (2025) reports, over 11.1% – or 1 in 9 – of the adult population (20-79 years) is living with diabetes, with over 4 in 10 unaware that they have the condition. By 2050, projections show that 1 in 8 adults, approximately 853 million, will be living with diabetes, an increase of 46%. Both type 1 and type 2 diabetes mellitus can cause severe complications in the nervous and cardiovascular systems, including cognitive decline and cardiac insufficiency, respectively. Among the different types of diabetes, over 90% of people with diabetes have type 2 diabetes [9]. Persistent hyperglycemia in diabetes progressively damages multiple organs, such as the kidneys, heart, liver, and eyes, contributing to increased mortality [10]. Uncontrolled diabetes results in vascular changes and dysfunction, and diabetic complications are the major causes of morbidity and mortality in diabetic patients. Among diabetic vascular complications, nephropathy is a leading cause of end-stage renal disease and an independent risk factor for cardiovascular diseases. Renal inflammation is recognized as one of the important pathophysiological mechanisms and therapeutic targets for the prevention of diabetic nephropathy and atherosclerosis [11].

Animal studies indicate that diabetes induces inflammatory and fibrotic changes in the liver [12]. CR, when combined with a balanced diet, has been shown to reduce albumin excretion, enhance creatinine clearance, and prevent collagen accumulation in the kidneys [5]. In diabetic Wistar fatty rats, dietary restriction ameliorated diabetic nephropathy [5], while in Emory mice, CR (at 60–79% of ad libitum intake) delayed cataract onset and progression and extended the median lifespan by 40% [6]. These effects could be attributed to the anti-inflammatory properties of CR. Additionally, CR reduces growth factor signaling, oxidative stress, and cognitive decline, slowing aging in rodents, monkeys, and humans alike [13]. Studies in rhesus monkeys demonstrate that CR delays age-associated pathologies, including diabetes and CVD [8]. In humans, CR improves metabolism and lowers serum C-reactive protein, TNF-α, and carotid intima-media thickness [14], suggesting that its protective vascular effects stem from reduced vascular inflammation—a key factor in diabetic nephropathy and atherosclerosis [11, 15].

Additionally, several studies have highlighted the neuroprotective benefits of CR, in particular related to sirtuin proteins. These sirtuin proteins play a key role in neuroprotection and aging-related cognitive decline. Research on neurodegeneration and sirtuin proteins has shown that CR increases brain sirtuin protein levels [16]. Neuroinflammation, cognitive function, particularly in conditions of obesity-related brain dysfunction are also ameliorated by CR. This effect is partly attributed to increased GABA levels in the

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hippocampus, which help mitigate inflammation and enhance neuronal function [17]. Similarly, memory, attention, and processing speed, are also improved with moderate CR, by reducing neuroinflammation and oxidative stress while increasing brain-derived neurotrophic factor [18].

Studies show CR has been explored as an intervention strategy for type 2 diabetes and metabolic dysfunction. A clinical trial investigating the effects of dapagliflozin plus CR for diabetes remission found that CR alone led to diabetes remission in 28% of participants, while combining CR with dapagliflozin increased remission rates to 44%. This suggests that while CR is beneficial, it can be further enhanced with pharmacological interventions [19]. On comparison between intermittent versus continuous CR for metabolic dysfunction-associated steatotic liver disease (MASLD), both strategies are effective in improving liver health, indicating that intermittent fasting may be a viable alternative to traditional CR for managing metabolic disorders [20].

The role of CR in promoting longevity has also been investigated. CR, in combination with metformin and GdVO4:Eu3+ nanoparticles, improved survival rates and physiological parameters by reducing oxidative stress and enhancing the prooxidant-antioxidant balance. However, strict CR in elderly individuals may have limitations due to long-term feasibility concerns [21]. Collectively, these studies suggest that CR provides health benefits not only in healthy individuals but also in those at risk for or affected by metabolic disorders.

Studies show that CR enhances pancreatic beta-cell function, improves glycemic control, increases insulin sensitivity in the liver and peripheral tissues, and optimizes insulin secretion. However, few studies have explored the impact of CR on gene expression in diabetic models because gene expression is regulated by various factors and can be either upregulated or downregulated. Therefore, this study aims to investigate the effects of CR on the expression of apoptotic and inflammatory genes in a type 2 diabetic animal model.

Table 2 Biochemical and Other Parameters of the Animals in the Study

S. No.	Parameters	Control group	DM group*
1.	Body weight (g)	248.9±21.4	143.06±22.9
2.	Food intake (g/day)	15.3±2.04	26.2±3.7
3.	Water intake (ml/day)	23.3±3.1	97±9.1
4.	Urine volume (ml/day)	23±3.4	88.3±8.8
5.	Urine sugar (g/day)	0.1±0.01	7.7±1.4
6.	Fasting blood sugar (mg/dl)	90.5±4.5	422.9±32.8
7.	HbA1c	3.7±0.4	8.9±1.0

*- STZ-Induced Diabetic Group; DM- Diabetes Mellitus

II. MATERIALS AND METHODS

A. Animal Model and Induction of Diabetes

Male Wistar rats, were housed in the animal house facility (CFTRI, Mysore) with a 12-h light/dark cycle. They had free access to a rodent diet and water. All animal procedures were approved by the Institutional Animal Care and Use Committee, CFTRI, Mysore. Rats weighing about 150-160g male rats were given a single dose of STZ (45 mg/kg body wt; Sigma, St. Louis, MO) dissolved in sodium citrate buffer (0.1M, pH 4.5). Rats that served as vehicle controls were given the same volume of sodium citrate (0.1M, pH 4.5), intraperitoneally. The rats were provided with a 5% glucose solution in place of water for the first 48 h to prevent the initial STZ-induced hypoglycemic mortality. Whole-blood glucose obtained from rat tail snipping method was monitored using Accu-check glucometer (according to the manufacturers protocol). After 72h, fasting blood glucose levels were monitored; rats with whole-blood glucose > 250mg/dl were considered as diabetic and included in the experiment.

B. Tissue Processing and Isolation of Alveolar Epithelial Cells from Lung Tissue

Rats from both control and diabetic group were euthanized after anesthetizing with Isoflurane (SRL chemicals). Lungs were dissected and washed with 0.15M NaCl and antibiotic antimycotic solution. The lung tissue was minced in protease solution containing 0.25% trypsin sterile in 133mM NaCl, 5.2mM KCl, 1.89mM CaCl2, 1.29mM MgSO4, 2.59mM Phosphate buffer, pH 7.4, 10.3mM HEPES buffer (pH 7.4), and glucose 1mg/mL and filtered through 150µm and 30µm nylon mesh. The filtrate was centrifuged at 1500 rpm for 10min at room temperature. The pellet was dissolved in DMEM supplemented with 10% FBS and 1% antibiotic and antimycotic solution and plated in T75cm2 cell culture flask and incubated at 37°C, 5% CO2 and observed for the formation of epithelial and or fibroblast monolayer.

C. Real-time Quantitative PCR

Total RNA was extracted from cells isolated from the control lung and the diabetic lung tissue using Genex RNA extraction kit, as per the manufacturer's protocol. DNA-free

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RNA was reverse-transcribed into cDNA using Verso cDNA synthesis kit (Thermo Scientific). Real-time PCR was performed on the Biorad CFX96 Real Time PCR machine using SYBR Green I as a double-stranded DNA–specific dye according to the manufacturer's instructions (Biorad). The PCR reaction mix contained 2µL of diluted cDNA, 10µL of SYBR green master mix, 0.5µL of10µM forward and reverse primers and 7µL of nuclease-free water in 20µL reaction volume. Optimal Primer concentrations and annealing temperature were determined by preliminary experiments. The PCR conditions used were as follows: 40 cycles of denaturation at 95°C for 15 s, annealing for 30 s, and extension at 72°C for 45 s followed by melt curve. The normalized gene expression levels of the target genes in different groups were calculated using the 2-ΔΔCt method. 18S rRNA was used as the reference gene in the analysis.

Cells were cultured in the growth medium supplemented with 5mM and 25mM glucose concentrations for 3, 8, and 10 days. At the end of the respective incubation periods, cells were examined for phenotype changes. Cells were subsequently lysed, and the total RNA was extracted and analyzed for inflammatory and apoptotic gene expression analysis by real-time quantitative PCR. The primers used in this study is shown in table 1.

III. RESULTS AND DISCUSSION

At 3 months post-injection, STZ-injected animals showed hyperglycemia (fasting blood sugar 423mg/dl Vs. 91mg/dl), glycosuria (7.7g/day Vs. 0.01g/day), polyuria (88ml/day Vs. 23ml/day), polydipsia (97ml/day Vs.23ml/day), polyphagia (26g/day Vs. 15g/day), decreased body weight (143g Vs. 249g) and increase in the percentage of glycated haemoglobin (9% Vs. 4%) compared to the controls (as depicted in Table 2). The values in the parenthesis represent mean values calculated from six animals. The above data confirmed that STZ-induced rats were diabetic.

The alveolar epithelial cells from both groups were isolated by density gradient centrifugation and subsequently cultured in the growth medium for 7 days. Cells isolated from the diabetic lung were heterogeneous with small patches of epithelial cells, and major population of spindle shaped, elongated cells resembling the fibroblast phenotype (Fig1).

It is known that a concentration of 5mM glucose in the growth medium represents the fasting blood glucose levels in-vitro in organisms and hence has been used as an in-vitro model to study the hyperglycemia induced pathological changes in cells [22]. To examine the effect of CR on cellular changes, cells isolated from the diabetic rat lung (that had

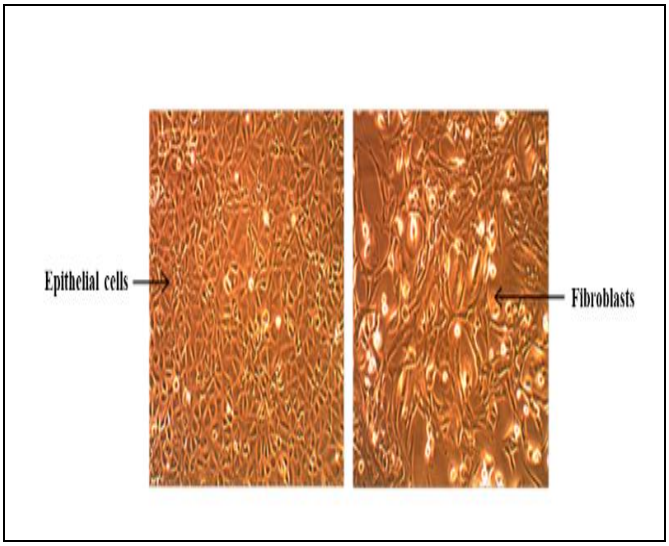

Fig 1 Control and Diabetic Cells Observed for Phenotype Changes

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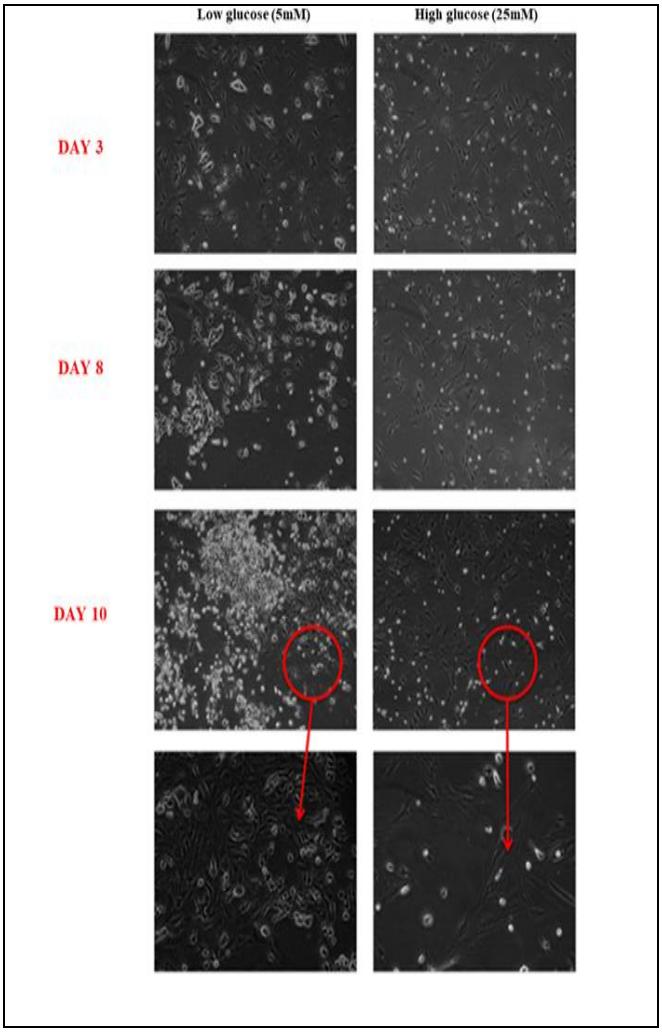

Fig 2 Comparison of Calorie Restricted and Non-Calorie Restricted Lung Cells

been exposed to in-vitro hyperglycemia environment) were cultured in the growth medium containing 5mM and 25mM glucose concentration. On day 3 and 8 post culture, cells cultured in growth medium in 5mM glucose concentration showed epithelial like-cell morphology. On day 10, patches of epithelial cells were observed together with small population of cells having the fibroblast phenotype (Fig2).

On the other hand, cells cultured in 25mM glucose concentration retained the fibroblast morphology even after 10 days post culture (Fig2). These results indicate that lowering the glucose levels (CR) in the medium promoted the transition of epithelial phenotype while higher glucose concentration mediated the retention of fibroblast phenotype. These findings suggested that CR can ameliorate hyperglycemia-induced pathological changes in cells. However, the mechanism for the development of the fibroblast phenotype in the lung cells in higher glucose concentration is not known.

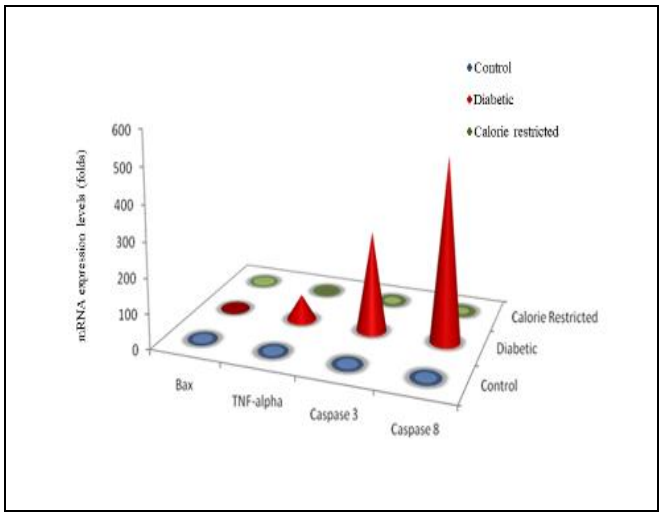

Fig 3 Expression Levels of Apoptotic Cells

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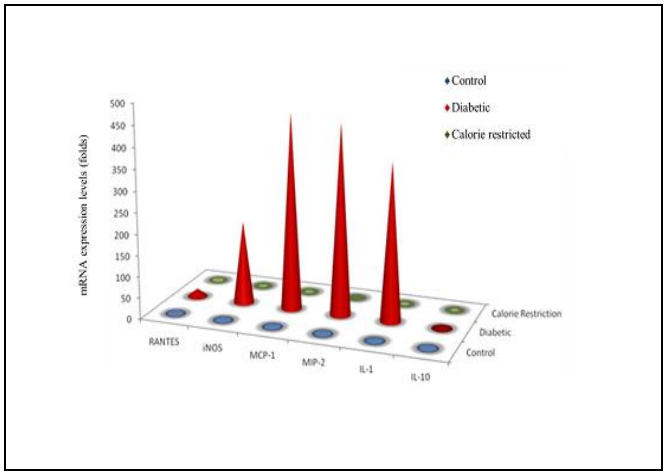

Fig 4 Expression Levels of Inflammatory Cells

It is possible that hyperglycemia may contribute to the lung fibrosis through the activation of advanced glycation end products (AGEs)-activated signaling pathways [23] or through the induction of oxidative stress pathways [24].

To understand how CR promotes the transition to the epithelial phenotype from the fibroblast phenotype, the expression levels of apoptosis and inflammatory genes by real-time quantitative PCR in the cells isolated from the diabetic lung were first examined. It was found that expression levels of apoptotic genes such as bax, TNF-α, caspase 3 and caspase 8 were substantially up regulated in the diabetic cells compared to that in controls (Fig 3) suggesting that diabetes induced pathological changes in the lung were associated with the induction of apoptosis.

Further, data also indicated that diabetes-induced apoptosis was mediated through the activation of caspase-dependent apoptotic pathways. To investigate if diabetes-induced pathological changes were also associated with the induction of inflammatory changes, the expression levels of inflammatory genes were also examined. Data revealed that diabetes also induced the substantial up regulation in the expression levels of pro-inflammatory cytokine genes such Rantes, iNOS, MCP-1, MIP-2 and IL-1 compared to that in controls (Fig 4). However, the expression level of anti-inflammatory cytokine such as IL-10 was not altered in both control and diabetic cells. To verify the effect of CR on the expression levels of apoptosis and inflammatory genes, diabetic cells were cultured in 5mM glucose concentration and subsequently examined for the changes in the above gene expressions.

As shown in Fig 3 and 4, CR significantly down regulated the expression levels of both apoptosis and inflammatory genes and was comparable with the control. These findings suggested that diabetes-induced apoptotic and inflammatory changes were mediated through hyperglycemia. CR can alleviate these hyperglycemia-induced pathological changes in the lung and promotes transition to epithelial phenotype from the fibroblast phenotype. However, the mechanism by which glycemia mediates the expression of apoptosis and pro-inflammatory genes is not well understood. It is reported that CR down regulates the induction of oxidative stress associated with hyperglycemia and can also activate the expression of activity levels of anti-aging molecules such as sirtuins in cells.

These sirtuins have been found to mediate the activity of various transcription factors including NF-κB, PPARs and FoxOs contribute for cell longevity and increased lifespan [25].

IV. CONCLUSION

CR, since being first proposed as a factor in longevity, extensive research has explored its various health benefits. Reducing calorie intake without micronutrient deficiency has been shown to enhance health at both systemic and cellular levels. Inflammation is a known trigger of apoptosis, and CR has emerged as a potential intervention for mitigating diseases associated with increased inflammation and cell death.

This study demonstrates that CR influences cellular health at the genetic level under diabetes-induced stress. Elevated glucose concentrations exacerbate oxidative stress, leading to increased expression of inflammatory and apoptotic genes. Whereas, CR mitigates these harmful effects, suggesting its potential to counteract diabetes-related cellular damage. These findings indicate that moderate CR may be particularly beneficial for individuals with type 2 diabetes mellitus, especially those with a genetic predisposition to inflammation. Nevertheless, further research is needed to validate and expand upon these results.

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