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EFFECTS OF DIET ON CCL11 EXPRESSION AND AGING OF LUNGS AND TESTES OF MICE

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ABSTRACT

Aging is a complex phenomenon symbolized by dynamic loss of tissue homeostasis with diminished cellular function. Along with genetic factors, many epigenetic factors as such lifestyle of an individual also affects aging. The aim of this study was to check the effect of different food habits in the process of aging using an aging biomarker CCL11. An increase in expression of CCL11 was observed in carbohydrate and fat rich foods groups. However, no effect on CCL11 expression was seen in calorie restricted diet groups. Mice fed with carbohydrates rich food gained weight, showed high levels of glucose, HDL and triglycerides while consumption of fat rich food in mice raised serum levels of anti-nuclear antibodies and LDL comparing to the control groups. Different food habits affected differently the serum levels of glucose, anti-nuclear antibodies, and lipid profile. H and E staining of testis and lungs showed that consumption of fat rich food caused emphysema, epithelial degeneration of lungs and aspermatogenesis in testes. Moreover, Calorie restricted diet showed vacuolization in testis. This study shows that food influence aging, expression levels of CCL11, physiology and morphology of lungs and testes in mice.

Key words: Aging, CCL11, epigenetic factor, aspermatogenesis, dietary habits
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INTRODUCTION

Aging can be defined as manifestation of various deleterious changes in the cells and tissues which increase the risk of death and disease (Thompson *et al.,* 2010 ; Da Silva, *et al*., 2021). The process of aging progressively influences reproductive, metabolic, physical, and intellectual capacity and eventually survival of an organism. Hallmarks of aging include genetic instability, telomere degradation, cellular aging, stem cell exhaustion, and altered intercellular communication (Lopez-Otin *et al.,* 2013; Ivanovska *et al.*, 2020; David *et al*., 2021) . Evolutionary studies have suggested that aging not only occurs because of gene programming but also by environmental factors and food habbits. Different studies have shown that intake of fat rich food triggers the inflammatory responses and causes high risk of cardiovascular diseases. (Villeda and Wyss-Coray, 2013; Guerville *et al.,* 2020).

Trans fats can cause inflammation which induce aging process by facilitating deterioration of telomeres (Kummerow, 2009). The shortened length of chromosomes directly refers to accelerated aging process (Lombard *et al.*, 2005); Guo *et al*., 2022). Excessive sugar intake can cause problems by attaching to protein molecules; this process is called glycosylation which induce earlier aging by several ways including augmenting oxidative stress which leads to the accumulation of toxins known as AGEs which refer to advanced glycation end products (Wolff *et al.*, 1991; Kim *et al*., 2017). The accumulation of such toxins is normal with advancement of age but due to excess intake of sugars their ratio increases fivefold and cause acceleration of aging process. This increase in age related toxins damage cell's power house mitochondria and leads towards the loss of cellular energy and cause many age related clinical outcome including memory loss, vision impairment and hearing loss etc. (Eckert *et al*., 2003). In humans and rodents, the aging process, induced by food habits, is associated with many structural, chemical, physiological and functional changes.

According to recent studies it has been reported that with an increase in the age of an organism, there are distinct changes in the expression of different genes (Shavlakkadze *et al*., 2019). One of such genes is the chemokine, CCL11 (Eotaxin) which belongs to CC chemokine subfamily encoded by gene located on chromosome 17q12 (Menzies-Gow *et al.,* 2002). The serum level of CCL11 chemokine is elevated when a person advances in age hence the serum level of CCL11 can be used as a biomarker of aging (Tacke *et al.*, 2007; Liang *et al.,* 2018). One of the most studied areas in aging is inflammation as various studies have shown that there is an increase in the production of cytokines, inflammatory markers, like interleukins, tumor necrosis factors and CCL11 which is an important chemokine (Sarkar and Fisher, 2006; Hoefer *et al*., 2017; Ivanovska

et al., 2020) . Serum profiling of these markers can tell much about their level of expression in normal and aged tissues. Elevated level of CCL11 has a profound effect on various body tissues because receptors for CCL11 are constitutively expressed in the thymus and inducibly expressed in the lung, intestine, heart, spleen and kidney and reproductive organs (Romieu and Trenga, 2001). This study was conducted to check the effect of food on CCL11 expression and related changes on aging process in lungs and testes of mice.

MATERIALS AND METHODS

Experimental Animals: All experiments were performed on male BAL B/C albino mice, age 6-8 weeks with an initial average weight of 37.66gm. All animals were raised in the animal house of Institute of Microbiology and Molecular Genetics (MMG), University of the Punjab at constant temperature (24 ± 2C) in natural light-dark cycle (12-12 h.). Mice were fed with standard diet and water. For this study, Institutional guidelines of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA were followed (Guide for the care and use of Laboratory Animals: Eighth Edition, 2011). A prior approval was also taken by Institutional Biosafety and Ethical Committee of MMG, University of the Punjab, Lahore.

BALB/C albino mice were randomly divided into six groups on the basis of type of food fed to them and each group consist of 6 mice. The groups include, control group (cannabis along with normal diet), normal aging group (aged mouse with age of more than 2 months), normal diet group, carbohydrates rich food group (soft drink which contained 10mg carbohydrates per 100ml along with their normal feed), fats rich food group (coconut milk, butter and cheese), and a calorie restriction group that was kept on starvation cycle for 24 h for 8 weeks.

Determination of Blood Glucose Level: Blood was collected from all the experimental groups by cardiac puncture by directly inserting needle into the heart and blood glucose level was measured at the time of dissection using Glucometer (ACCU CHEK Active).

Quantification of CCL11 By ELISA: Amount of CCL11 in the blood was quantified by enzyme linked Immunosorbent assay (ELISA) using a commercially available Eotaxin (CCL11) Mouse Simple Step ELISA™ Kit (abcam; catalog number ab201277) **(**Mansoor *et al.*, 2017).

Quantification of Anti-Nuclear Antibodies By ELISA: The blood levels of anti-nuclear antibodies were determined by enzyme linked immunosorbent assay (ELISA) using commercially available kit Catalog number GA-E0266MS (Mansoor *et al.*, 2017).

Lipid Profiling: Complete lipid profile of all the mice was done in which the serum level of total cholesterol, Triglycerides, HDL, LDL was determined by enzymatic colorimetric method using a, commercially available reagent kit (Spectrum diagnostics) for the quantitative determination (Haemmerle *et al.*, 2002).

Histological Structure of Lungs and Testes: The tissues were fixed in Bouin's fixative later embedded in paraffin wax following routine histological technique. Sections (3um) were stained with Hematoxylin and eosin (Cardiff *et al*., 2014).

Statistical analysis: The statistical analysis was carried out using the Prism graph pad 6.0 version. One-way ANOVA was used to assess the data. P value less than 0.05 was considered as significant. No significant difference (ns), $^{***}P < 0.001$.

RESULTS

Mice fed with carbohydrate rich food gained weight and showed hyperglycemic effect: Generally, the weight of mice increased in carbohydrate rich food group while there was no considerable change in weight of mice fed with normal food. On the other hand, mice kept on a starvation cycle for 24 h followed by 24 hours of feeding, mice given fat rich food and mice given cannabis with their normal feed showed a decrease in weight. Weight of the mice was recorded at the beginning and end of the experiment for comparison. Moreover, considerable variation in glucose level was observed among different groups of mice as compared to control groups. The group of mice given carbohydrate rich food have elevated glucose levels (291.6 mg/dL) as compared to a normal diet (227.3 mg/dL) and normal aging groups (215.7 mg/dL). The group of mice fed with fat rich food showed reduced levels of glucose and those fed with cannabis and starvation group showed almost normal level of glucose (226.3 mg/dL) in their blood as can be seen in Fig. 1.

Carbohydrate rich food causes increase in blood HDL and triglyceride level: The amount of HDL in the group of mice provided with carbohydrate rich food (53.3 mg/dl) was high comparing to a slight increase in the group provided with fat rich food, cannabis with the normal diet group and starvation group (49.01 mg/dl) as can be seen in Fig 2d. Furthermore, the amount of triglycerides was increased in the group of mice provided with carbohydrate rich food (243.6 mg/dl) and a slight increase in the starvation group and in the group of mice provided with cannabis (176.36 mg/dl) Fig. 2b.

Fats consumption cause increase in blood levels of LDL: The highest level of LDL was observed in the

group provided with fat rich food (93.26 mg/dl) and no significant increase in the other groups was seen. Fig. 2c. As higher intake of carbohydrate than the body requirements increases cholesterol level therefore total cholesterol level was higher in carbohydrate rich food group (183mg/d1) comparing to the control groups (135 mg/dl) Fig. 2a.

Carbohydrates and fats rich foods induce early aging while starvation and cannabis group have anti-aging effect: The concentration of chemokine CCL11 was noticed among different groups of mice. The serum concentration of CCL11 in carbohydrate and fat (2662.52 pg/ml) group was higher as compared to normal aging (1713.75 pg/ml) group which leads to induction of early aging. While concentration of CCL11 in group of mice with calorie restriction was reduced (1255.68pg/ml) as compared to normal aging group. Cannabis in the food of mice caused decrease in concentration of CCL11 (1133.256 pg/ml) showing an anti-aging effect Fig. 3.

This data was expressed as mean + SEM (n = 5 per group). The data was analyzed by applying One Way ANOVA and *t*-test. Group of mice given carbohydrate rich food has shown elevated level of glucose as compared to normal diet and normal aging group $^{*}p<0.05$ vs. normal diet group, $^{*}p<0.05$ vs. normal aging. Group of mice given fat rich food showed decreased glucose level as compared to control (cannabis) groups$^{**}p>0.05$ vs. control group.

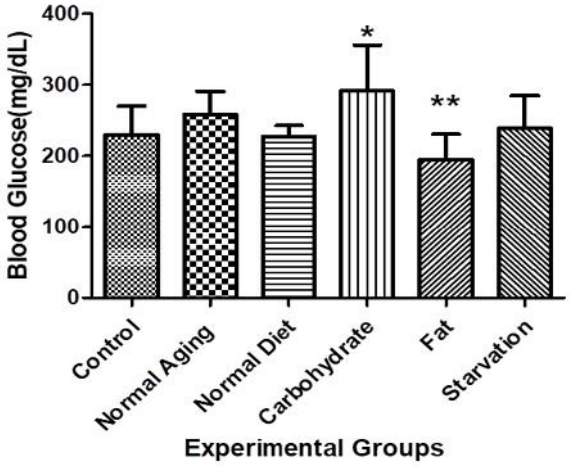

Figure 1: Hyperglycemic and hypoglycemic effect of different foods in mice.

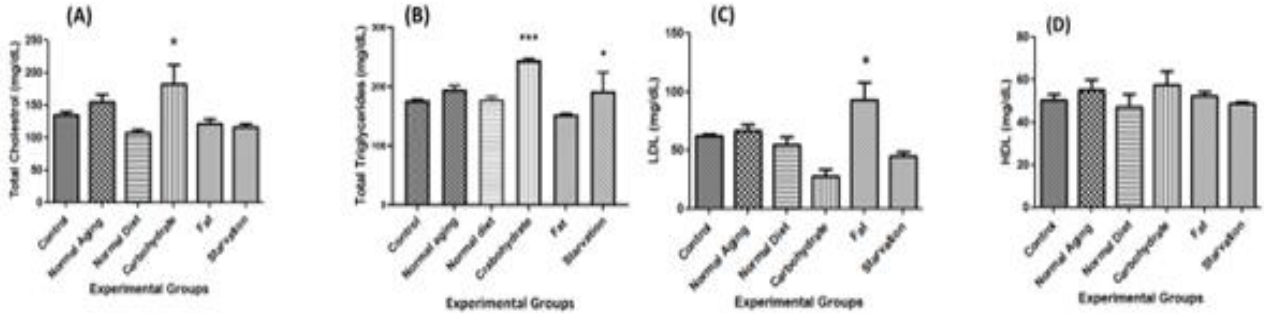

Figure 2: Effect of different diets on Lipid Profile of mice in each diet group.

(A) Total cholesterol. Higher level of total cholesterol was observed in group given carbohydrate rich food $^{*}p< 0.05$ vs. control (cannabis) group. It can be correlated with excessive intake of glucose **(B)** Triglycerides. Group of mice kept on starvation cycle of 24 hours also showed elevated TC $^{*}p<0.05$ vs. control (cannabis) group. **(C)** LDL. Our results showed increased level of LDL in group of mice given fat rich food which clearly indicates a direct relation between LDL level and high fats intake $^{*}p<0.05$ vs. control group. **(D)** HDL. No significant increase or decrease in HDL level as compared to control groups while group given carbohydrate rich food showed slight increase in HDL. This data was expressed as mean + SEM (n = 5 per group). The data was analyzed by applying One Way ANOVA and *t*-test.

These results showed that increased level of CCL11(pg/ml) in the group of mice provided with carbohydrates rich food and fat rich food as compared to the normal diet group. Significant difference was noted $^{*}p<0.05$ vs. normal diet group and $^{**}p<0.05$ vs. normal diet group. Significant decrease in CCL11 was observed in starvation group $^{***}p>0.05$ vs. normal diet group. This data was expressed as mean + SEM (n = 5 per group). The data was analyzed by applying One Way ANOVA and *t*-test.

Fat rich food enhances serum level of anti-nuclear antibodies in mice: Group given with fat rich food and the group given cannabis with their food showed elevated levels of anti-nuclear antibodies (198.485 pg/ml). While the group fed on carbohydrate rich food and mice given food for 24 hours, followed by 24 hours starvation showed almost normal level of ANA (161.97 pg/ml) Fig. 4.

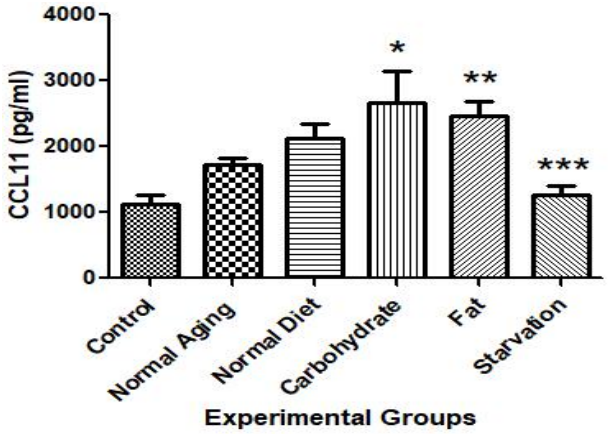

Figure 3: CCL11 quantities pg/ml in mice serum provided with different types of foods.

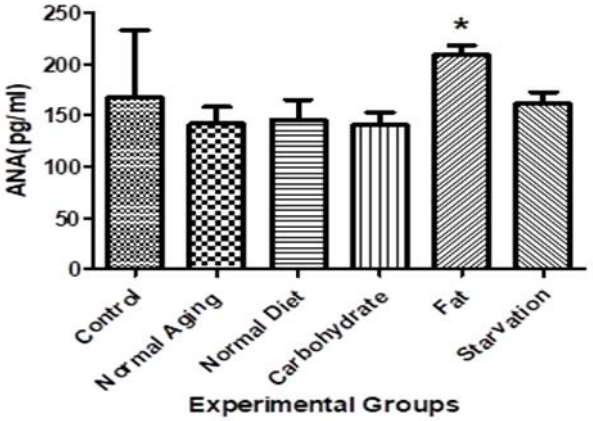

Figure 4: Effect of different dietary habits in the production of Anti-Nuclear Antibodies (ANA) in the mice.

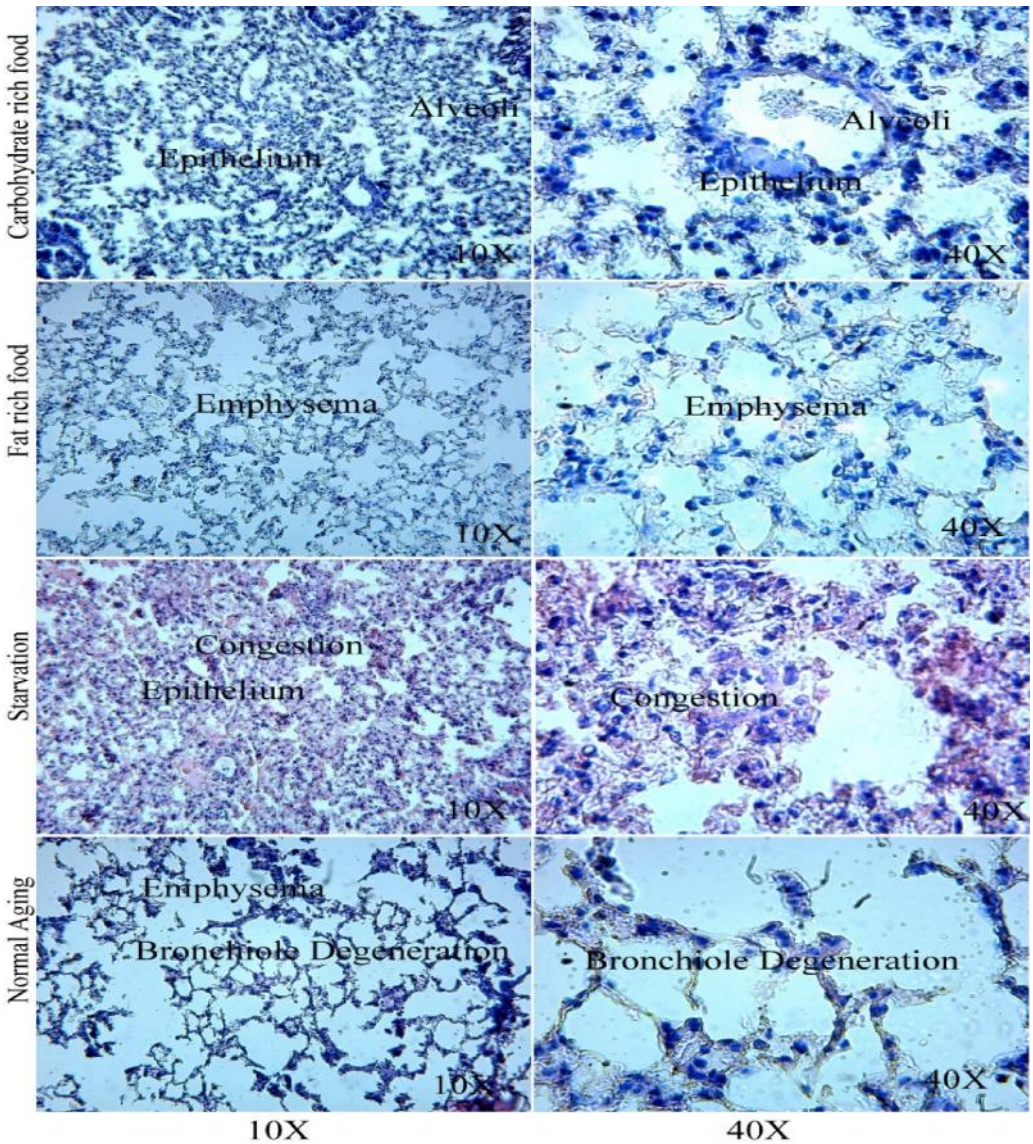

Figure 5: Histological structure of lungs of mice.

This result showed that higher levels of anti-nuclear antibody (ANA) were observed in the group of mice provided with fat rich food $^{*}p< 0.05$ vs. control (cannabis) group. This data was expressed as mean + SEM (n = 5 per group). The data was analyzed by applying One Way ANOVA and *t*-test.

Fat rich food and cannabis cause emphysema and epithelial degeneration in lung tissues of mice: Starvation and fat rich food caused emphysema in lung tissue. Emphysema is damage of air sacs causing shortening of breath. Cannabis caused epithelium degeneration, emphysema and pulmonary fibrosis. Pulmonary fibrosis is the scarring of tissue deep in lungs and it causes lungs to become thick and stiff. Intake of carbohydrate rich food showed no considerable change in morphology of lung tissue. Normal aging group showed emphysema, epithelium degeneration and congestion in alveoli Fig. 5.

Images taken at 10X and 40X magnification showing emphysema in tissues of mice given fat rich food and congestion in starvation group in comparison to control group in bottom panel.

Fat rich food cause aspermatogenesis in mice while starvation cause vacuolization in testes of mice: Fat rich food given to mice caused fat deposition in the tissues and aspermatogenesis. Intake of carbohydrate rich food showed no considerable change in morphology of testes tissue. The group of mice which were aged according to their normal developmental pattern showed reduced spermatid production. Starvation caused epithelium degeneration in the male reproductive tissue (testes) of mice, which leads to retrogressive changes in the function of the tissue. Vacuolization which is small vacuole formation and aggregation of spermatids was also observed in the tissues of the starvation group Fig. 6.

Images taken at 10X and 40X magnification showing fat deposition at various sites in tissues of mice given fat rich food and epithelium degeneration, vacuolization in starvation group along with epithelial degeneration and reduced spermatids in control group.

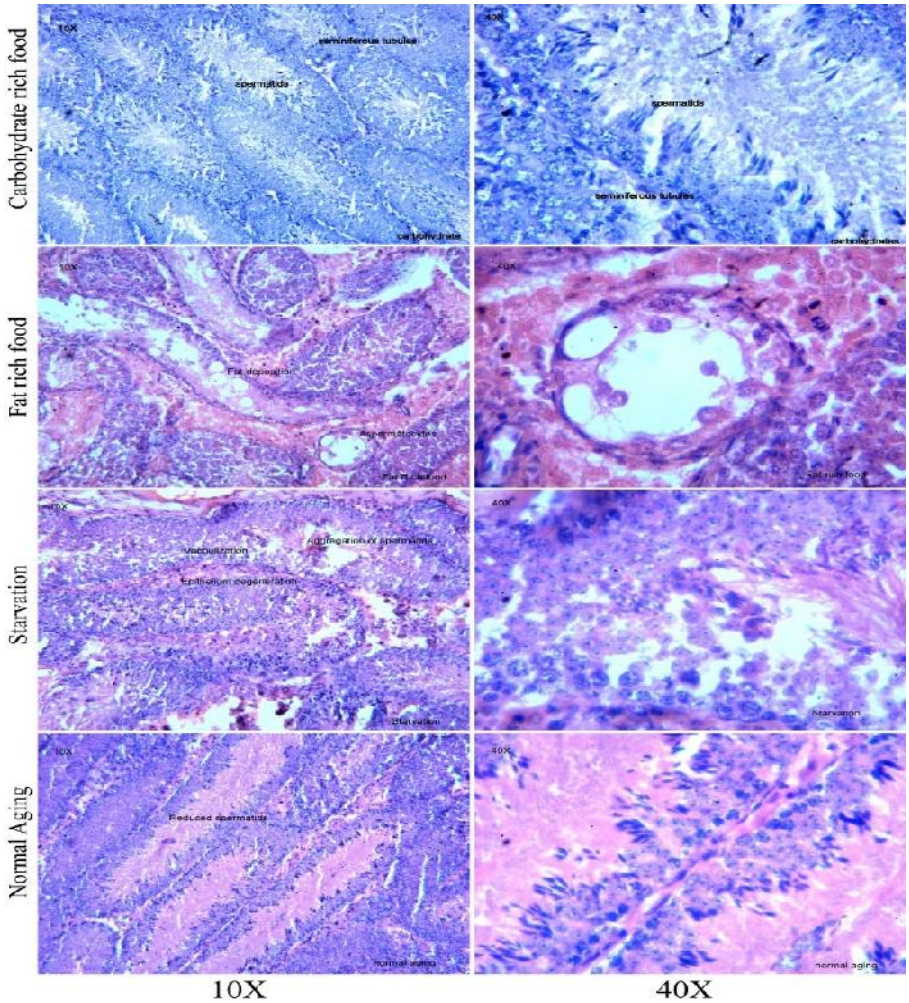

Figure 6: Histological structure of testes of mice

DISCUSSION

Aging is a progressive loss of mechanisms of homeostatic pathways which are necessary to maintain structural and functional characteristics of an organism (Thompson *et al.*, 2010; Zia *et al*., 2021). Alterations in the physiology of an organism's body are majorly due to genetic impact. However, epigenetic factors play a major role in aging. Food is the basic source of nutrients for an organism. Different nutritional components are associated to the process of aging and contribute to affect the life span of an individual (Masoro, 1988; Picca *et al.,* 2017). The purpose of our study was to check the effect of different dietary habits on the process of aging. As there are several molecules which are involved in aging, we can use these molecules as biomarkers of aging. We selected a major chemokine: CCL11 also known as Eotaxin. It is the most important chemokine which expression is directly correlated with aging (Wyss-Coray *et al.,* 2011).

Carbohydrate rich diet showed increased glucose level in the serum whereas replacing it with mono-saturated fat rich diet has decreased blood glucose level (Garg *et al.*, 1988). Aging can be associated with decrease in blood glucose tolerance (Chen *et al.*, 1988 ; Kalyani *et al*., 2013) and as seen in carbohydrate rich diet, the level of glucose is higher and the level of CCL11 is also higher. Decreased blood glucose level in the group of mice provided with fat rich food showed that there were no carbohydrates i.e., glucose and therefore the metabolic pathway shifted towards fat metabolism converting fats into smaller subunits (glycerol and fatty acids) entering the citric acid cycle for energy production (Manninen *et al*.,2004).

Furthermore, lipid profiling was also performed to check the quantities of different types of lipids in blood of all different diet groups. In our study, it was seen that total cholesterol, triglycerides, and HDL level was increased in carbohydrate rich food group comparing to the control group while LDL was increased in fat rich food group comparing to the control group. Previous studies reported that high intake of sugar is linked with unfavorable blood lipid and glucose concentrations (Aumeuller *et al*., 2021). Therefore, there should be a balance of sugar and fats in food as rise in both of these cause non-communicable disease and mortality (Valencia *et al*., 2021).

Lower level of CCL11 production in the starvation group indicated that there is no induced aging in this group. It is already studied that calorie restriction leads to less production of reactive oxygen species (ROS) which is considered as one of the most important cause of early aging (Guarente, 2008; Bianchi *et al*., 2016). The increased levels of CCL11 (pg/ml) in the group of mice provided with a carbohydrates rich diet indicated that there was an induced early aging in high carbohydrate diet. Specifically, high sucrose diet can lead to obesity (Storlien *et al.*, 1988; Ahmed *et al*., 2019) and studies performed on obese mice showed higher level of oxidative stress in them (Furukawa *et al,*. 2004). Oxidative stress can cause DNA damage and expedite telomerase shortening which unnecessarily induces aging (Metcalfe *et al*., 2012).

Higher levels of anti-nuclear antibody were observed in the group of mice provided with fat rich food. A study reported that high fat diet leads to autoantibody production and pro-inflammatory cytokines (Peeta *at al*., 2018; Fernandes *et al.*, 1973). The dysregulation in cytokines itself leads towards aging (Rea *et al*., 2018) along with anti-nuclear antibody (Fernandes *et al.*, 1973). Restriction of diet can prevent several age-related diseases and can delay the process of early aging (Shammas *et al*., 2012).

Food and nutritional factors have a great impact on activities and functioning of reproductive tissues. The onset of obstructive reproductive problems is mainly due to intake of a particular diet in excess or improper intake, which can cause a decrease or increase in inflammatory reactions. Inflammation has a direct relation with aging (Singh *et al*., 2011) and the expression of CCL11, which recruits eosinophil at the site of inflammation (Romieu and Trenga, 2001).

Tissue processing results have shown no abnormality in the morphology of reproductive and lung tissues of the normal diet group and the group given carbohydrate rich food. The tissues of normal aging group showed reduced spermatids production which indicates that with the progressive age, there is decline in the reproductive function of testis. The process of aging affects testes for their endocrine function i.e. production of testosterone is reduced, which is involved in spermatogenesis (Feldman *et al.,* 2002; Gunes *at al*., 2016).

Epithelial degeneration and vacuolization were observed in tissues of the group of mice kept on starvation of 24hour cycle. It can be due to hypoxia inducible factors and stress conditions (Sitkovsky *et al.,* 2004). Group of mice fed with fat rich food showed emphysema and severe epithelial degeneration in lung tissues. Moreover, fat deposition and decreased spermatogenesis was observed in testes. In previous studies, it is shown that intake of fat rich food has a major impact on lung tissue. Reasons behind such problems can be reduced production of carbon dioxide by fat metabolism which causes reduced ventilation in alveoli as reported (Efthimiou *et al.*, 1992). The group of mice given cannabis with normal food showed fibrosis in lung tissue: which is thickening and scarring of tissues around alveoli. The reason behind fibrosis caused by cannabis is still unknown (Phan *et al.,* 2005).

Hence, our study shows that food influence aging, expression levels of CCL11, physiology and morphology of lungs and testis of mice.

Conclusion: This study concludes that the expression of selected biomarker, CCL11, is affected by dietary habits of an individual. Hence food has a huge impact on process of aging causing increase in expression of CCL11 and affecting the morphology and physiology of body tissues such as lungs and testis.

Conflict of interest: The author(s) declare that there is no conflict of interest.

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